Development of tropical freshwater mussel toxicity tests and an assessment of key contaminants from the Ranger uranium mine

A thesis submitted in fulfilment of the requirements for the degree of

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed. I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

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Abbreviations

ACR	Acute-to-chronic ratio
AEC	Animal Ethics Committee
ANOVA	Analysis of Variance
ARR	Alligator Rivers Region
$CaSO_4$	Calcium sulfate
Cu	Copper
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
EC10	Concentration causing a sub-lethal effect to 10% of test organisms
EC50	Concentration causing a sub-lethal effect to 50% of test organisms
eriss	Environmental Research Institute of the Supervising Scientist
HEPES	Buffer (N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid)
LC50	Concentration causing lethality to 50% of test organisms
MCW	Magela Creek Water
MES	Buffer (4-Morpholineethanesulfonic acid)
Mg	Magnesium
$MgSO_4$	Magnesium sulfate
mtDNA	Mitochondrial DNA
NH3	Un-ionised ammonia
NH_4^+	Ionised ammonia
NOEC	No observed effect concentration
SSD	Species sensitivity distribution
TAN	Total ammonia nitrogen
TOC	Total Organic Carbon
U	Uranium
UO_2OH^+	Hydrated uranyl ion
UO_2^{2+}	Free uranyl ion

List of publications included in this thesis

Chapter 3

1. Kleinhenz LS, Trenfield MA, Mooney TJ, Humphrey CL, van Dam RA, Nugegoda D, Harford AJ. 2018. Acute ammonia toxicity to the larvae (glochidia) of the tropical Australian freshwater mussel *Velesunio* spp. using a modified toxicity test protocol. *Environ Toxicol Chem* 37(8):2175-2187. DOI: 10.1002/etc.4175.

Chapter 4

2. Kleinhenz LS, Humphrey CL, Mooney TM, Trenfield MA, van Dam RA, Nugegoda D, Harford AJ. 2019. Chronic ammonia toxicity to juveniles of two tropical Australian freshwater mussels (*Velesunio* spp.): toxicity test optimization and implications for water quality guideline values. *Environ Toxicol Chem* 38(4):841-851. DOI: 10.1002/etc.4370.

Chapter 5

3. Kleinhenz LS, Nugegoda D, Trenfield MA, van Dam RA, Humphrey CL, Mooney TJ, D, Harford AJ. 2019. Acute and chronic toxicity of magnesium to the early life stages of two tropical freshwater mussel species. *Ecotoxicology and Environmental Safety* 184 (2019) 109638. DOI: 10.1016/j.ecoenv.2019.109638.

Conference presentations during candidature

1. **Kleinhenz L,** Nugegoda D, Trenfield M, van Dam R, Humphrey C, Harford A. Development of an acute and chronic toxicity test for the freshwater mussel *Velesunio angasi*, and an assessment of ammonia toxicity. Oral presentation at SETAC - AU (Society of Environmental Toxicology and Chemistry - Australasia) Conference, 4 – 7 October 2016, Hobart, Australia.

2. **Kleinhenz L,** Nugegoda D, Trenfield M, van Dam R, Humphrey C, Harford A. Chronic toxicity of ammonia to the tropical freshwater mussel *Velesunio* spp., Oral presentation at SETAC - AU (Society of Environmental Toxicology and Chemistry - Australasia) Conference, 4 – 6 September 2017, Gold Coast, Australia.

3. **Kleinhenz L,** Nugegoda D, Trenfield M, van Dam R, Humphrey C, Harford A. Development of toxicity testing protocols to assess contaminants of potential concern to freshwater mussels. Oral presentation at the inaugural ASSSS (Annual Supervising Scientist Science Symposium), 29th Oct 2018, Darwin, Australia

4. **Kleinhenz L,** Nugegoda D, Trenfield M, van Dam R, Humphrey C, Harford A. The freshwater mussel, *Velesunio* spp., is a valuable test species for assessing the acute and chronic toxicity of contaminants in tropical environments. Oral presentation at SETAC - AU (Society of Environmental Toxicology and Chemistry - Australasia) Conference, 7 – 10 July 2019, Darwin, Australia.

Abstract

Many aquatic species are at risk from the toxic effects of anthropogenic stressors. Benthic invertebrate species may be more vulnerable to water-borne and sediment-bound contaminants due to their sedentary lifestyle and feeding habits, which may result in multiple routes of exposure. One such vulnerable species is the freshwater mussel, which is both a filter feeder and a deposit feeder in its early life stages. Mussel larvae (glochidia) are exposed to contaminants in the water column or during their obligate parasitic attachment stage on the host fish, while juvenile mussels are exposed to dissolved and suspended contaminants in surface waters, sediment and pore water through their filtering activity or ingestion via pedal feeding.

The contamination of mussel habitats is one of many natural and anthropogenic stressors implicated in the increasing global decline of freshwater mussel species, particularly in North America. Research continues but is still lacking on the taxonomic status and sensitivity to contaminants of Australasian species, particularly those in tropical habitats. Freshwater mussels are widely distributed throughout tropical Northern Australia, including creeks and billabongs of high conservation value surrounding the Ranger uranium mine lease. Here, mussels play important roles both ecologically and culturally, and extensive research focusing on the biology and ecology of the most common species, *Velesunio angasi*, has been previously undertaken. A monitoring program for bioaccumulation of radionuclides and metals has been carried out annually using *V. angasi* since 2000 due to the importance of mussels in the diet of Aboriginal communities downstream of mine water discharges. After 40 years of operation, the Ranger mine is scheduled to cease operations in 2021. However, the risk of contamination from mining waste is expected to continue following rehabilitation activities, through mobilisation and leaching from contaminant sources on the mine site. Contaminants of potential concern (COPCs) from the Ranger mine include ammonia, magnesium, and uranium, and a toxicity testing program is in place to predict the toxicity of these COPCs and inform the water quality objectives for the mine. Toxicity data for the COPCs are generated primarily using local tropical species, but have also incorporated other suitable international toxicity data, to derive site-specific water quality guideline values for the slightly acidic and extremely soft waters of the catchments downstream of the mine. Such water quality generally causes increased metal bioavailability and toxicity. The water quality objectives aim to protect the World Heritage-listed downstream ecosystems, and the local

Aboriginal people that rely on them, from the effects of uranium mining. However, to date, freshwater mussels have not been included as a test species due to culturing difficulties and a lack of standardised toxicity test methods.

A preliminary and secondary aim of this thesis was to confirm the genetic identity of freshwater mussels collected from populations both within and outside of the Alligator Rivers Region (ARR), Northern Territory, that could potentially be used for toxicity testing. Adult mussels were sampled from 13 different sites, and a 710-bp fragment of the mitochondrial COI gene was amplified and sequenced from the mantle tissue of each mussel (~15 mussels per site). Phylogenetic analysis revealed three distinct clades containing three known species and three to four unknown, or undescribed, species. The billabong (or lentic) water-body sourced species matching to *Velesunio angasi* were placed in Clade A, and diverged by 11.1% from the lentic creek-sourced mussels in Clade B that did not match to any known species. The lotic *V. angasi* and the lentic unknown species, referred to as *Velesunio* sp. throughout this thesis, were subsequently selected for the toxicity testing research. Mussel collection sites were chosen based on factors such as accessibility of the two species, proximity, and relevance to the Ranger mine.

The primary aims of this thesis were to optimise acute and chronic toxicity testing protocols for use with tropical mussel species, and conduct toxicity testing using the optimised test protocols. Test optimisation was undertaken using guidance from previous work with *V. angasi* and an ASTM International standard guide. Acute toxicity test optimisation involved testing different variables including glochidia selection, pH control, test volume, test vessels, number of glochidia per replicate, age of glochidia, and test duration. Following test optimisations, 24-h acute, water-only toxicity tests were undertaken using mussel glochidia from both species with ammonia, magnesium, copper, and uranium, using survival as the endpoint. Development and optimisation of the chronic test protocol involved refinement of a host fish exposure method (in order to produce juvenile mussels) using two different fish species, trialling different test volumes, the addition of fine sediment <63 µm (silt), food and feeding frequency, and biological endpoints. Using the final protocol, 14-d chronic toxicity tests with ammonia, magnesium, and uranium were undertaken on newly-released (<24-h) juvenile mussels that had been metamorphosed from glochidia in the laboratory using the host fish, *Mogurnda mogurnda*, using growth rate as the sub-lethal endpoint. All toxicity tests were conducted at pH 6.0 ± 0.3 and 27 ± 0.5 °C to represent local environmental conditions.

Velesunio spp. glochidia and juveniles were found to be similarly and highly sensitive to ammonia, with mean median lethal (LC50) acute toxicity estimates ranging from 7.4 to 11.9 mg/L total ammonia nitrogen (TAN), and mean median effect (EC50) chronic toxicity estimates ranging from 7.0 to 11.3 mg/L TAN. When toxicity estimates were normalised to pH 7 and 20°C, *Velesunio* spp. were among the most sensitive species to ammonia in comparison to temperate freshwater mussel species and other tropical taxa. *Velesunio* spp. glochidia were also highly sensitive to copper, with LC50s ranging from 5.2 to 8.4 µg/L, and similarly sensitive in comparison to temperate freshwater mussel species. Acute and chronic exposures to magnesium indicated that *Velesunio* spp. were moderately sensitive, with LC50s ranging from 284 to 300 mg/L, and mean EC50s ranging from 232 to 241 mg/L. For acute uranium exposures, LC50s ranged from 227 to 375 µg/L, indicating moderate sensitivity in comparison with acute data for other tropical taxa. When developing the chronic uranium exposure method, it was demonstrated that chronic uranium exposures using pre-spiked sediment did not significantly reduce the loss of uranium from the water column, and control growth was reduced in comparison with tests using un-spiked sediments. Addition of un-spiked sediments produced satisfactory control growth in the chronic exposures, but nonetheless, water chemistry measurements indicated a significant loss of U to the sediment, with filtered U concentrations reduced by up to 90% between the start and end of tests. Median EC50s for the filtered and total fractions of the three un-spiked sediment tests ranged from 64 to 126 µg/L U, and 276 to 506 µg/L U, respectively. When comparing LC50s and EC50s for each contaminant tested, intra- and interspecies variability was low (≤2-fold), indicating similar sensitivity within and between *V. angasi* and *Velesunio* sp.

This thesis contributes new knowledge of freshwater mussel genetic diversity in Northern Australia, and provides efficient and robust toxicity testing methods for tropical freshwater mussels. The acute and chronic toxicity data generated using the optimised test methods are a valuable contribution to the tropical datasets for the contaminants of concern. The data can be used to inform site-specific and regionally relevant water management practices for the protection of *Velesunio* spp., and the freshwater ecosystems they inhabit.

CHAPTER 1: Introduction and review of the literature

1.1 Freshwater mussels

Freshwater mussels are important components of aquatic environments throughout the world. They provide valuable ecosystem services such as bio-filtration, nutrient transformation, and transfer of matter and energy from the water column to the benthic environment (Lopes-Lima *et al.* 2017). In addition to these regulating and supporting roles, mussels are an important food source for animals and humans (Vaughn 2017). Their abundance, sessile nature, long lifespan, filter-feeding habit, and high bio-concentration ability for contaminants make them an ideal indicator organism for environmental pollution (Beiras *et al.* 2003). In lowland rivers, the presence of freshwater mussels has been positively correlated with taxon richness of other invertebrates (Aldridge *et al.* 2007). A study on the mollusc communities in Lake Winnipeg, Canada indicated that mussel species richness was positively correlated with total dissolved solids, and negatively correlated with anthropogenic lead concentrations (Pip 2006). Due to their sedentary life habits, freshwater mussels are known to be vulnerable to degraded water and sediment quality. Being benthic filter and deposit feeders, juvenile and adult mussels may be exposed to contaminants via multiple pathways through surface water, sediment, and pore water by ingesting filtered particles containing sorbed contaminants (Augspurger *et al.* 2007).

1.1.1 Distribution and taxonomy

Freshwater mussels are an ancient group of bivalve molluscs comprising six families within the order Unionoida (Figure 1.1). The Unionidae and Margaritiferidae families are most common in the Northern hemisphere, while the Hyriidae family is most common in the Southern hemisphere (Walker *et al.* 2014). The global diversity of freshwater mussels is currently estimated at 927 species in 179 genera (Graf and Cummings 2018). The Unionidae are by far the largest and most widespread family, consisting of approximately 717 known species, more than 300 of which occur in North America (Graf and Cummings 2007; Graf and Cummings 2018).

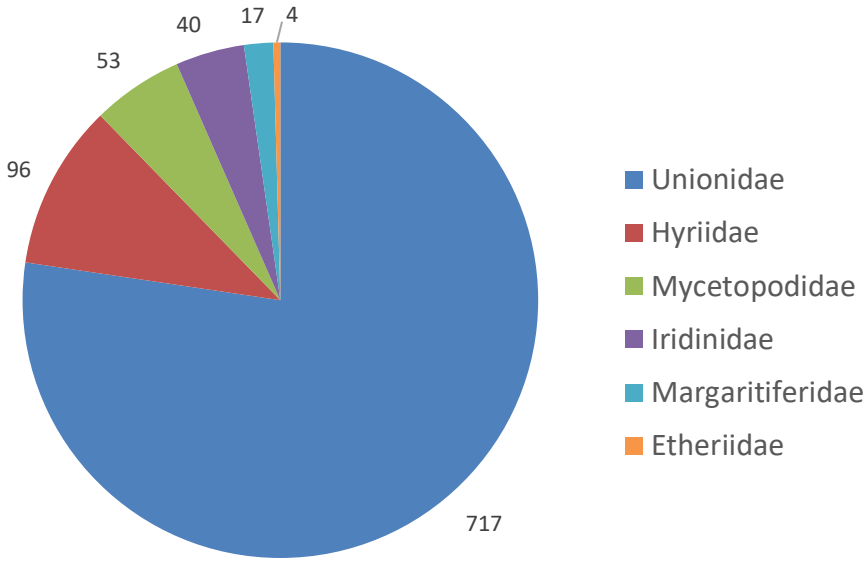

Figure 1.1: Family level diversity of the 927 freshwater mussel species within the six Unionoida families. The number of species are shown for each family (data from Graf and Cummings 2018).

In Europe, 16 species (2 Margaritiferidae, 14 Unionidae) are currently recognised (Lopes-Lima *et al*. 2017). In the Australasian ecozone (Australia, New Zealand, the Solomon Islands, and New Guinea), 31 of approximately 32 known species belong to the Hyriidae family (Graf and Cummings 2007; Walker *et al*. 2014). Within the Australian Hyriidae, the two subfamilies, Hyriinae and Velesunioninae, are differentiated by shell characteristics and glochidia (larval) morphology (Walker *et al*. 2014). Of the five *Velesunio* species within the Velesunioninae, two occur most commonly in tropical northern Australia: *Velesunio wilsonii*, and *Velesunio angasi* (Figure 1.2; Walker *et al*. 2014).

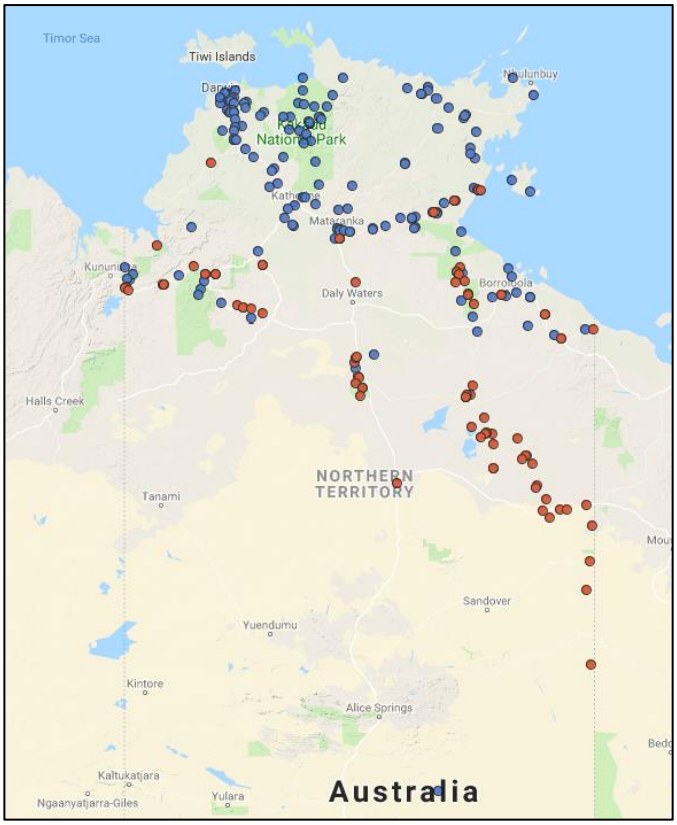

Figure 1.2: Occurrence records of two mussel species, *Velesunio angasi* (blue circles) and *Velesunio wilsonii* (red circles) in the Northern Territory, Australia (map generated from ALA 2019).

Traditionally, the taxonomy for freshwater mussels has been based on morphology of larvae above the family level, and morphology of shells and soft tissues at genus and species levels (Baker *et al*. 2004). However, taxonomic identification using morphological methods and, in particular shell characteristics, can be unreliable due to the high degree of morphological variability and convergence seen within and between species. Molecular approaches such as DNA bar-coding are a powerful alternative tool for providing reliable identification of adult mussels and their larvae (Zieritz *et al*. 2012). In North America, molecular methods of mussel identification are well established, but this is not the case for Australian species where diversity is lower (Baker *et al*. 2004). The identification of Australian mussel species is difficult in Hyriidae due to the lack of genetic data. Molecular techniques in combination with morphometric data have been recommended for improving knowledge on the diversity and evolution of Australian freshwater mussels (Baker *et al*, 2003). Indeed, three cryptic species were discovered in central Australia using these techniques (Baker *et al*, 2003; Walker *et al*. 2014). Currently, data on the population and taxonomic status of Australasian species is deficient, and there is a need for comprehensive

and systematic revision of Australian freshwater mussel fauna to clarify the nature and relationships of species (Walker *et al.* 2014).

1.1.2 Conservation status

Worldwide, freshwater mussel populations have been experiencing a decline in numbers for several decades. Many factors are implicated in reducing mussel populations, including sedimentation, habitat alteration from construction of dams, disease, oxygen depletion, competition with exotic species, loss of host fish species, and environmental pollution from sewage, agriculture, or mining (Pip 2006; Augspurger *et al.* 2007; Graf and Cummings 2007).

Currently, around half of the 466 freshwater mussel species that have been assessed by the International Union for the Conservation of Nature have been listed as extinct, critically endangered, endangered, vulnerable or near threatened (Figure 1.3; IUCN 2019).

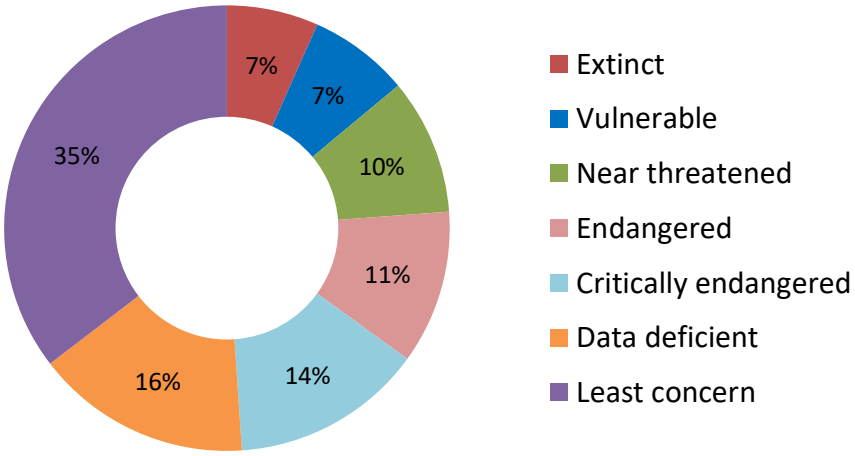

Figure 1.3: Global status of the 466 freshwater mussel species (data from IUCN 2019).

In North America, Unionid mussels are one of the most endangered faunal groups, with 35 extinct species, and 70% of species listed as endangered, threatened, or of special concern (Augspurger *et al.* 2003; Augspurger *et al.* 2007). In Europe, 12 of the 16 recognised species are listed on the IUCN Red List as critically endangered, endangered, vulnerable, and near threatened (Lopes-Lima *et al.* 2017), while in Asia, the conservation status of 61% of the 231 known species has not yet been assessed by the IUCN, due to a lack of data (Zieritz *et al.* 2018).

Among the 32 known Australasian freshwater mussel species, seven are currently listed as threatened (Walker *et al*. 2014). Chemical contaminants have been implicated frequently as a contributing factor in the decline of freshwater mussels globally, and published data indicate that mussel early life stages are highly sensitive to inorganic chemicals such as chlorine and ammonia, and some metals (Augspurger *et al*. 2003; Wang *et al*. 2007a) when compared to the most sensitive species of other invertebrates, fish or amphibians (ASTM 2006). Section 1.2 describes the effects of contaminants on freshwater mussels in more detail.

1.1.3 Life cycle

The fascinating and unique reproductive mode of freshwater mussels includes a brief obligate parasitic stage on a fish host, during which mussel larvae (glochidia) metamorphose into juvenile mussels (Kat 1984). The reproductive cycle begins when sperm released by the male into the water column are captured by the female through their incurrent siphon. Fertilisation takes place and the embryos develop into glochidia within modified chambers of the female's gills (marsupia). Once mature, large numbers of glochidia are either released into the water or may be retained until suitable conditions arise (Wang *et al*. 2007a; Jacobson *et al*. 1997). After release, the critical obligate parasitic stage must occur within a few days to complete development. The success of glochidial attachment to the gills and fins of the fish host is reliant on the incidence of fish-host contact and the method of parasitism, which varies greatly between mussel species (ASTM 2006). Most species of Unionidae (including Hyriidae) release free glochidia into the water, but some have developed complex mechanisms and adaptations that increase their chances for attracting host fish (Barnhart *et al*. 2008), and are associated with their evolutionary diversification (Watters 2007). For example, a wide variety of mantle lures are displayed in the genus *Lampsilis.* As the host fish attempts to eat the lure (sometimes an extension of the mantle tissue resembling a small fish), mature glochidia are released, which then attach to the fish (Zanatta and Murphy 2006). The endangered fanshell pearly mussel, *Cyprogenia stegaria*, releases conglutinate packages containing mature glochidia which resemble worms, and are also mistaken by the fish for prey (Jones and Neves 2002).

Of the three morphological forms of glochidia, a hooked, triangular form, is more prevalent than the unhooked D-shaped form or the "pick-axe" form (Watters 2007). Australian Hyriidae are of the hooked form, with distinctive sub-triangular valves and S-shaped hooks (Walker *et al*. 2014). Although general observation has indicated that hooked forms are more specialised for attachment to the outside surfaces of the fish host and

unhooked forms are usually gill parasites (Watters 2007), Humphrey and Simpson (1985) observed that glochidia of the tropical Australian freshwater mussel, *Velesunio angasi* (Hyriidae), were more commonly attached to the gills rather than the fins.

If successful in attachment to the fish host, glochidia are rapidly encapsulated in a layer of host fish tissue. This transformation stage typically lasts between two and six weeks, although significant variations occur with different water temperatures, mussel species, and host fish species (Cope *et al*. 2008). After metamorphosis, juvenile mussels detach from the host fish to the substratum, becoming free-living members of the benthic community (Jacobson *et al*. 1997). Juveniles are thought to typically burrow below the sediment surface, feeding on bacteria, algae and detritus in the pore water of sediment using foot (pedal) feeding to draw particles into the mantle cavity. Adults are sedentary filter feeders, feeding on suspended particles such as phytoplankton from the water column (ASTM 2006).

1.2 Toxicity testing with freshwater mussels

Aquatic ecotoxicology studies include those that measure the effects of chemicals on aquatic organisms at various levels, including subcellular, individual organisms, populations, and whole ecosystems (Moiseenko 2008). Controlled laboratory experiments using single species continue to be a valuable source of data for assessing sensitivity to contaminants. However, Chapman (2002) argued that to adequately protect populations against contaminants and other stressors, improvements need to be made in combining ecology and toxicology. It has also been increasingly acknowledged that the value and quality of some ecotoxicological research needs improvement (Harris *et al*. 2014). A set of reporting requirements and broad recommendations were proposed by Hanson *et al*. (2017) to improve the reporting of ecotoxicology studies based on their reliability and relevance, and ultimately improve the quality of the science available to regulators.

Mussels are considered good indicator species in environmental toxicology for several reasons, including their relative sensitivity (March *et al.* 2007), limited mobility (Besser *et al.* 2015) and amenability to toxicity testing in the laboratory (Augspurger *et al.* 2007). Toxicity tests are carried out on the sensitive early life stages of freshwater mussels (glochidial stage and juvenile stage), and the adult life stage to provide experimental evidence about the effects of a particular contaminant, in order to develop water quality guidelines for the protection of freshwater species (Ingersoll *et al.* 2007).

In response to the global decline of freshwater mussels and their generally high sensitivity to some contaminants, research on culturing and toxicity test methods have

increased within the past two decades. The lack of standard toxicity testing protocols was hindering the development of chemical-specific water quality criteria (Cope *et al*. 2008), and hence, the American Society for Testing and Materials (ASTM) *Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels* was published in 2006 to provide guidance on culturing methods and to address the lack of standard toxicity testing protocols. Specific components of the standard covered ecologically relevant test duration, test acceptability criteria, toxicity endpoint selection, and procedures to ensure health of the mussels in laboratory culture and testing (ASTM 2006; Augspurger *et al*. 2007). As a result, the use of standardised methods has increased the number of published toxicity data for freshwater mussels; and confirmed their high sensitivity to a range of common metal contaminants and major ions including copper, nickel, zinc, lead, ammonia, sulfate, and chlorine (March *et al*. 2007; Wang *et al*. 2007a; Kovats *et al*. 2010; Gillis 2011; Wang *et al*. 2017), in comparison to other freshwater taxa. One limitation of the ASTM (2006) standardised methods is that their development was based on toxicity tests using temperate freshwater mussel species, specifically North American species (Unionidae). Mussel species belonging to other families and from different climatic regions may differ in their culturing and toxicity testing requirements, and test optimisation may be required to accurately assess their relative sensitivity to contaminants. For example, survival times of glochidia of the Margaritiferidae were shown to be shorter than those of other Unionoida families when tested in waters of the same temperatures, and glochidia viability decreased in all families as water temperatures increased (Akiyama and Iwakuma 2007). Increased temperature not only influences the biological effects on test organisms including rates of metabolism, uptake, and detoxification, but can influence the water chemistry of test systems. Differences in sensitivity to contaminants between temperate and tropical aquatic organisms need to be considered in the toxicity testing methodology to accurately reflect environmental risk (Kwok *et al*. 2007).

1.2.1 Acute toxicity testing

Acute tests are typically conducted on glochidia for 24 to 48 h, and on juvenile mussels for 96 h, using survival as the endpoint. In toxicity tests with glochidia, viability of glochidia at the start of the test is determined by observing the closure of mussel valves in response to a concentrated salt solution. This method is also widely used to measure the survival endpoint (also termed ‘viability’ in some studies; ASTM 2006). The response of glochidia to a salt stimulus is thought to be an indicator of their ability to attach to a host fish in the natural

environment, and is therefore considered ecologically relevant (Fritts *et al.* 2014). Whilst exposure to fish plasma or other salts could be used to assess glochidia, sodium chloride (NaCl) is considered more practical to work with (Ingersoll *et al*. 2007). Fritts *et al*. (2014) found that using NaCl to measure glochidia response was a good indicator of their subsequent capacity to attach to a host fish and metamorphose into juvenile mussels, providing the control viability was >90%. They recommended that the current ASTM guideline using the valve closure response to NaCl be retained, and that toxicity tests should be conducted on newly-released glochidia to minimise loss of viability.

Several techniques have been described in the literature for removal of glochidia from gravid females. Many studies have removed glochidia by flushing the female mussel gills with a syringe, as recommended by ASTM (2006). This method has the advantage of being able to use the freshly released glochidia immediately in toxicity tests, and allows more precise determination of the age of glochidia following release. However, it is more suited to mussels that can be prised open without harm to the adductor muscles. Humphrey (pers. comms) placed female *V. angasi* in a shallow container of water and glochidia were aborted spontaneously as a stress response, without harm to the adult mussels. The neurotransmitter serotonin is known to regulate bivalve reproduction, and has been used successfully to induce spawning in zebra mussels (Fong 1998) and for the stimulation of synchronous glochidial release in *Hyriopsis bialatus*, a cultured mussel from Thailand (Meechonkit *et al.* 2012). The use of serotonin to induce spawning has been researched for its potential in the improvement of *in vitro* larval culture (Meechonkit *et al.* 2012) and to help mitigate the spread of the invasive Quagga mussel (Schwaebe *et al*. 2013). Nonetheless, further research would be required to determine the effects of serotonin on the quality of released glochidia, and its use may be significantly cost prohibitive (Fong 1998).

1.2.2 Chronic toxicity testing

Chronic toxicity tests have been conducted on juveniles for 21 to 28 d, measuring the survival and growth endpoints (ASTM 2006). Growth is generally determined by measuring the increase in shell length or shell surface area, and gives a good indication of the sub-lethal effects of contaminants. Other endpoints such as dry weight (Wang *et al.* 2011), valve movement (Markich *et al*. 2000), clearance rate (Salerno *et al.* 2018) have also been measured. Chronic data are lacking for freshwater mussels, due to the challenges involved in culturing juvenile mussels in the laboratory for use in toxicity testing. Revision of the ASTM (2006) standard is ongoing, particularly for the refinement of chronic test conditions.

Uncertainties over appropriate chronic test durations, feeding regimes, endpoints, and appropriate starting age of juvenile mussels are still being addressed. For example, recent work by Wang *et al*. (2018b) refined a feeding regime for juvenile *Lampsilis siliquoidea* for use in 12-wk exposures of different starting ages (~1 wk to 2 mo) to sodium chloride (NaCl), following concerns that the ASTM (2006) recommended 4-wk exposure may not represent an adequate portion of the juvenile life stage, and that newly-transformed juveniles may be more sensitive to some contaminants than the recommended 2-mo mussels (Wang *et al*. 2007a). Younger juveniles were found to be more sensitive to NaCl than older juveniles, but the longer test duration did not substantially affect NaCl toxicity. To date, refinements of chronic test conditions have not been undertaken with tropical freshwater mussel species, which may require different test conditions to temperate species.

Efforts for propagating and culturing unionid mussels in the laboratory have expanded in the last two decades, particularly in North America, for the purposes of replenishing endangered and vulnerable species (Barnhart 2006). A limiting factor is the reduced growth rate experienced in the laboratory when compared to natural growth rates. This has been attributed to inadequate diet and increased energy expenditure (Barnhart 2006). The addition of fine silt to culture water has been shown to benefit the growth and condition of juvenile mussels, by providing nutritional and physical support (Hudson and Isom 1984; Gatenby *et al*. 1996). The primary method of juvenile mussel propagation in the laboratory, referred to as *in vivo* propagation, is carried out by exposing natural fish hosts to mature glochidia collected from adult female mussels (Lima *et al*. 2012). Artificial propagation (*in vitro)* methods that bypass the need for using host fish have also been developed and refined in recent decades, and used successfully to date on 42 species of unionid mussels in the USA (Lima *et al*. 2012). Glochidia undergo metamorphosis in a Petri dish containing a mixture of artificial cell culture media, fish serum and antibiotics. However, more research is needed to optimise successful propagation (Lima *et al*. 2012). Toxicity tests conducted by Popp *et al*. (2018) demonstrated that *in vitro* propagated juvenile mussels (using artificial culture media) were generally more sensitive than their *in vivo* counterparts. They suggested that *in vitro* propagated mussels could potentially be used to conduct toxicity tests, but that more research was needed to define the health and condition relationship between the two propagation methods (Popp *et al*. 2018).

1.2.3 Deriving water quality guideline values

Water Quality Guideline Values (GVs), described in other jurisdictions as criteria, standards, benchmarks or thresholds, are developed nationally and internationally for the purpose of providing guidance for management and protection of aquatic ecosystems from the impacts of contaminants (ANZG 2018). Water quality GVs are ideally derived using a weight of evidence approach, which evaluates multiple lines of evidence such as field, dietary, geochemical and mesocosm data (Buchwalter *et al*. 2017; Suter *et al*. 2017). This process incorporates judgements on the data from the different lines of evidence to make an overall assessment about water quality and its management (Suter *et al*. 2017). Exceeding the GV for a toxicant indicates that an impact could potentially occur (ANZG 2018).

In Australia, site-specific or regionally relevant GVs are limited for many toxicants, and default GVs are often applied using toxicity data reported in the international literature. Default GVs are less likely to be relevant for all aquatic ecosystems, and GVs in Australia could be improved through the provision of regional toxicity data using test conditions tailored to the environment being evaluated (ANZG 2018).

In Australia and New Zealand, GVs predicted to protect at least 95% of species are typically applied to aquatic ecosystems deemed to be slightly to moderately disturbed, which represents the majority of ecosystems. Where the ecosystem condition is thought to be worse or better than this, then GVs that afford slightly lower (80% or 90%) and higher (99%) species protection are typically applied, respectively (van Dam *et al*. 2014; Warne *et al* 2018). For example, a 99% species protection GV may be applied to a location with high conservation and ecological value, while a lower protection level of 80% might be selected for a highly disturbed ecosystem (ANZG 2018; van Dam *et al*. 2014).

Warne *et al*. (2018) recommended the use of data from at least eight species when deriving GVs using the Species Sensitivity Distribution (SSD) approach (section 1.2.4), which replaced the previous minimum data requirement recommended by the ANZECC and ARMCANZ (2000) guidelines of data for at least five species from at least four taxonomic groups. This is largely due to limited data available for Australian species and is less stringent than USA or EU requirements (Warne *et al*. 2018). Other key changes included clarifying the definitions of acute and chronic toxicity tests. Clear definitions were considered critical, for example, when acute and chronic data were combined to generate water quality GVs. In the revisions, chronic toxicity tests with macro-invertebrate adults or juveniles were recommended to be 14 days or greater in duration. It has been noted that this applies to

temperate species, and exceptions would exist for species in tropical climates; exposures of tropical organisms may be of shorter duration since development tends to be faster than that of temperate species (Batley *et al.* 2014).

A paucity of tropical data has led to the practice of extrapolating toxicity values from temperate data, under the assumption that responses and distributions of tropical and temperate species are similar (Kwok *et al.* 2007). The use of extrapolation creates a margin of uncertainty because the relative sensitivities of tropical and temperate species vary in response to different chemicals. Kwok *et al*. (2007) found that temperate species were more sensitive than tropical species to most metals, while tropical species were more sensitive to ammonia, arsenic, zinc, phenol, and some pesticides. Mooney *et al*. (2019) compared the chronic toxicity of ammonia to six tropical Australian species in low ionic strength waters with available temperate ammonia data. Toxicity estimates for two species (a cnidarian and a fish) were among the lowest reported; however, differences in sensitivity were thought to be influenced by the low ionic strength waters rather than physiological differences, supporting the need for site-specific GVs.

1.2.4 Site-specific water quality guideline values

Because default GVs cannot adequately account for the many environmental factors affecting toxicity at a particular location, the value of developing site-specific GVs for the protection of high value ecosystems is well-recognised (van Dam *et al.* 2014). Where a lack of data exists, e.g. for tropical water bodies, site-specific toxicity estimates are needed to develop GVs for such ecosystems to provide an appropriate level of protection based on local conditions (ANZG 2018). Existing guidance for selecting and evaluating approaches for deriving site-specific GVs is currently limited, which may lead to inappropriate GVs being applied (van Dam *et al.* 2019). In Australia and New Zealand, local GVs are recommended over default GVs (ANZG 2018). An increasing awareness of the need for local water quality GVs has led to proposals for refinements to the methods of GV derivation (van Dam *et al.* 2014, 2019).

In Australia, the preferred and most commonly used method of deriving site-specific GVs is based on the use of SSDs, similar to the method used for deriving default GVs. In this method, data acquired for each species and the relevant toxicant is acquired from laboratory toxicity tests or field biological data, and plotted as a cumulative frequency distribution. Site-specific GVs are derived for the level of protection required.

1.3 The Alligator Rivers Region

The Alligator Rivers Region (ARR) is an area of almost 30,000 km^2 in the wet-dry tropics of Northern Territory, Australia. Two-thirds of the ARR is occupied by World Heritage- and Ramsar- listed Kakadu National Park, internationally recognised for its significant natural and cultural values (Figure 1.4; Sinclair *et al.* 2013). The region is also valued for its extensive mineral reserves, particularly uranium.

1.3.1 Uranium mining in the ARR

Uranium mining in the ARR began with the discovery of uranium deposits in the South Alligator Valley in 1953. Thirteen uranium deposits were mined between 1959 and 1965. Renewed demand for uranium resulted in the discovery of four major deposits: Ranger in 1969, Nabarlek and Koongarra in 1970, Jabiluka 1 in 1971, and Jabiluka 2 in 1973 (Spiers 2000). Currently the Ranger uranium mine is the only mine in operation (van Dam *et al*. 2002; van Dam *et al.* 2014), with Jabiluka and Koongarra remaining undeveloped, and Nabarlek ceasing operations in 1988 and undergoing rehabilitation.

The Ranger mine lease is an area of 78 km^2 within the ARR, and is surrounded by Kakadu National Park. Mine waters are discharged in a controlled manner during the wet season (November to April) into the seasonally flowing Magela Creek, the key aquatic receiving environment downstream of the mine site. An integrated assessment, research, and monitoring program was established by the Australian Government in the 1980s to ensure protection of the aquatic ecosystems and people within the ARR from U mining (Humphrey *et al*. 1999; van Dam *et al*. 2002). Mining and ore processing operations at Ranger commenced in 1981, and are required by legislation to cease by 2021, with progressive decommissioning in advance of rehabilitation by 2026 currently underway (Supervising Scientist 2015). A key research priority over the operational life of Ranger has been the development of site-specific water quality GVs for key mining contaminants of potential concern in Magela Creek, which have been incorporated into regulatory Water Quality Objectives. Similarly, such GVs will inform closure criteria for the mine, which are needed to help ensure the off-site environment remains protected once the site has been decommissioned. Over the past 40 years, the key contaminants of concern have been identified as ammonia, magnesium, manganese and uranium (Harford *et al.* 2015; Supervising Scientist 2018a, b, c; van Dam *et al.* 2010, 2017; see section 1.3.2). Toxicity testing for at least six local species (Table 1.1) is used along with other relevant data for deriving site-specific water quality GVs for mining contaminants.

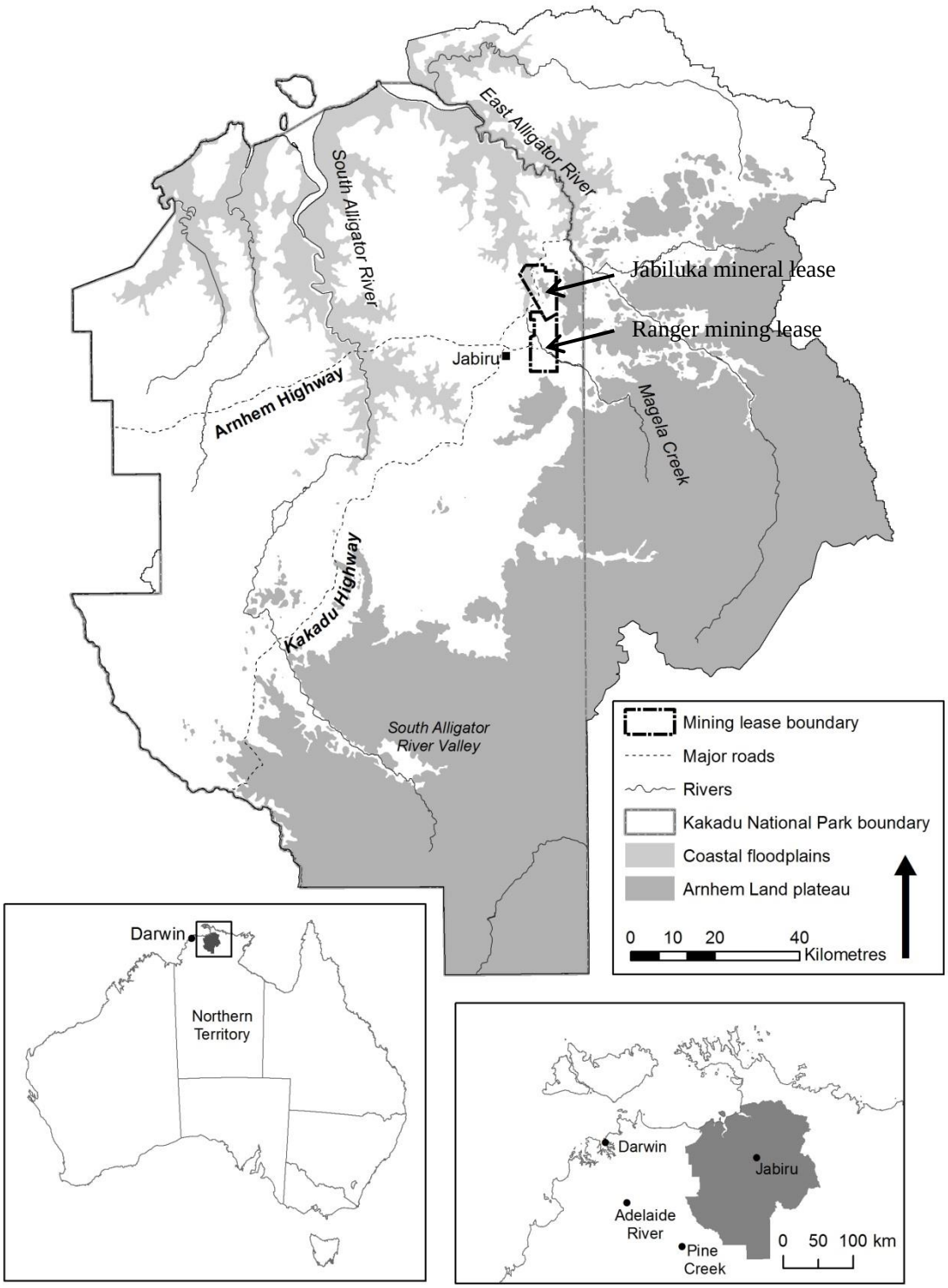

Figure 1.4: The Alligator Rivers Region, Northern Territory, Australia, showing the Kakadu National Park boundary (middle of picture), and the location of the Ranger uranium mining lease (map used with permission, Supervising Scientist 2015).

Table 1.1: Six local freshwater species used for toxicity testing at the Supervising Scientist.

Species	Common name	Phylum	Test type
Amerianna cumingi	Snail	Mollusca	96-h embryo production
Lemna aequinoctialis	Duckweed	Charophyta	96-h plant growth
Moinodaphnia macleayi	Cladoceran	Arthropoda	3 brood production
Hydra viridissima	Green hydra	Cnidaria	96-h population growth
Chlorella sp.	Green alga	Chlorophyta	72-h cell division rate
Mogurnda mogurnda	Fish	Chordata	96-h survival - acute

1.3.2 Contaminants of potential concern for the Ranger uranium mine

1.3.2.1 Ammonia

Ammonia is a common contaminant in aquatic environments due to its toxic nature and ubiquitous presence in surface water systems (Camargo and Alonso 2006). The natural formation of ammonia in the environment occurs during microbial fixation of atmospheric nitrogen and hydrogen, decomposition of organic material by bacteria, and in trace amounts produced by lightning (USEPA 2013). Ammonia is also produced anthropogenically through industrial waste and wastewater treatment plants, and from agricultural run-off from fertilisers and animal wastes (Augspurger *et al.* 2003). In the mining industry, ammonia is used extensively for the extraction of metals. Many effluents require treatment for removal of ammonia so that concentrations in receiving surface waters are maintained at an acceptable level (USEPA 2013). At the Ranger mine, ammonia is used during the final stages of uranium processing, to precipitate uranium from solution. Ammonia is present at concentrations of ~1000 mg L^{-1} in the process water, but does not currently pose a high environmental risk as this water type cannot be discharged untreated into the environment. However, future risks have been identified through the potential presence of ammonia in treated process water, and from possible seepage from the tailings and brine containment facilities after mine closure (Mooney *et al*. 2019).

The geochemical properties and interactions of different nitrogen species may have significant implications for the transport and fate of uranium and other trace elements at uranium mining sites (Miao *et al*. 2013). Two forms of ammonia exist in the aqueous environment, the more abundant ammonium ion (NH_4^+) and the less abundant non-dissociated or unionised ammonia (NH_3). The ratios of each depend on both pH and temperature. Generally, as values of pH and temperature increase, the concentration of NH_3

increases and the concentration of NH_4^+ decreases. The concentration of total ammonia (TAN) is analytically measured in water samples, and is the sum of NH_4^+ and NH_3 concentrations (USEPA 2013). Un-ionised ammonia (NH_3) often represents a small proportion of TAN, especially at pH 7 (Newton and Bartsch 2007), but for each rise of a single pH unit there is a 10-fold increase in the ratio of unionised ammonia to ammonium ion in fresh water (USEPA 2013).

Un-ionised ammonia (NH_3) is generally considered to be more toxic than ionised ammonia (NH_4^+) due to its neutral charge, which allows it to diffuse across cell membranes more readily (USEPA 1999). However, studies conducted with other freshwater species in dilution waters of low and ionic concentrations have demonstrated that NH_4^+ toxicity can contribute significantly to ammonia toxicity (Ankley 1995; Mooney *et al.* 2019). The toxic effects of ammonia that may contribute to freshwater mussel mortality include a reduction in the opening of valves for feeding and respiration (Epifanio and Srna 1975), negative effects on metabolism due to depletion of fat stores (Chetty and Indira 1995), and in some species, impaired ability to secrete the byssus thread for attachment to the substratum (Reddy and Menon 1979).

A general lack of ammonia data exists for tropical freshwater taxa, and much of the tropical ammonia dataset has been extrapolated from temperate data based on the assumption that sensitivity would be similar (Kwok *et al*. 2007). Similarly, most of the available ammonia toxicity data for freshwater mussels have been generated using temperate mussel species in North America and Europe, and prior to this study no ammonia toxicity data was available in the literature for tropical freshwater mussel species.

1.3.2.2 Magnesium

Magnesium (Mg) and sulfur (S) are both essential elements to organisms. Magnesium sulfate ($MgSO_4$) is a common contaminant in mining operations, and is associated with runoff from exposed ore, waste rock, and tailings. Until recently, $MgSO_4$ has received minimal focus as a contaminant when compared to other mining contaminants, although it is a key constituent in mine waters at the Ranger uranium mine (van Dam *et al*. 2010). Over 30 years of water quality data from this region have shown that Mg is the most likely mining solute to approach or exceed the guideline/limit values. Consequently, a detailed pulse exposure assessment framework for Mg has been developed in response to in situ monitoring of electrical conductivity demonstrating that environmental exposures were occurring in pulses (Sinclair *et al.* 2013).

In water bodies influenced by the Ranger mine, data has been obtained on the toxicity of magnesium sulfate ($MgSO_4$) to local species. It was found that discharges of $MgSO_4$ from the mine were positively correlated with increased electrical conductivity (or salinity) measured in the adjacent creeks, which raised questions about the effects of magnesium on the biota of Magela Creek (van Dam *et al*. 2010). In the same study, toxicity test results indicated that the toxicity of Mg was negatively correlated with calcium concentrations (which are already extremely low (~ 0.1 mg/L) in Magela Creek). A site-specific assessment of Mg was initiated, with a series of experiments using six local freshwater species. Magnesium was confirmed to be the toxic ion, and Ca was shown to have ameliorative effects on Mg toxicity. It was concluded that toxicity is dependent on Ca concentrations and that Ca deficient waters posed the greatest risk to aquatic life. Two site-specific water quality GVs were derived: 0.8 mg/L for waters with a Mg:Ca ratio of >9:1, and 2.5 mg/L for waters with a Mg:Ca ratio of ≤9:1 (van Dam *et al*. 2010).

In subsequent research, Hogan *et al*. (2013) quantified the effects of short-term pulses of Mg and found that toxicity increased as exposure duration increased for all six species tested. Further work on recovery time from magnesium pulses was recommended to provide guidance for the Mg/electrical conductivity guideline framework, considering the fluctuating nature of Mg in the creek systems (Hogan *et al.* 2013). A study was carried out on *Hydra viridissima*, a local green Hydra species sensitive to Mg (Prouse *et al.* 2015). The toxicity of multiple Mg pulses and inter-pulse recovery time was assessed and compared to single pulse recovery. Full recovery was observed for all Mg concentrations, pulse duration and recovery times tested, indicating that *H. viridissima* benefited from inter-pulse (recovery) periods irrespective of duration and timing. Recovery occurred faster than expected when compared to single-pulse recovery, and *Hydra viridissima* appeared to be less sensitive to multiple short Mg pulses than to a longer pulse of equivalent exposure. It was concluded that time-weighted averaging of exposure concentrations could provide a conservative method of predicting *H. viridissima* response (Prouse *et al*. 2015). To date, few magnesium toxicity data have been reported for freshwater mussels in temperate regions, and no acute or chronic data are available for tropical freshwater mussels.

1.3.2.3 Uranium

Uranium (U) is a naturally occurring radioactive metal that is mined for use as nuclear fuel. Uranium has 16 known radioactive isotopes, but in nature consists of a mixture of three: ^{238}U, ^{235}U, and ^{234}U. While U occurs naturally in freshwater in trace concentrations ranging from 0.01 to 6.6 µg/L, metal releases into the environment from mining activities can cause an increase in background levels and subsequent risk to aquatic biota (Gascoyne 1992). Uranium induces both chemical and radioactive toxicity in the environment, but chemical toxicity has been demonstrated to present a much greater risk (Mathews *et al*. 2009).

The bioavailability of U to aquatic organisms depends on its physiochemical form. In surface waters U may occur in three oxidation states: U^{4+} (U[IV]), UO_2^{+}(U[V]), and UO_2^{2+} (U[VI]. Dissolved organic carbon, pH, alkalinity and water hardness can all affect the speciation and bioavailability of U in various ways (Markich 2002; Trenfield *et al.* 2011b). Uranium is known to form complexes with inorganic (e.g. carbonate and phosphate) and organic (e.g. dissolved organic matter) ligands, reducing its bioavailability and thus toxicity (Riethmuller *et al.* 2001; Charles *et al*. 2002; Markich 2002; Hogan *et al*. 2005; Trenfield *et al*. 2011b). The free (UO_2^{2+}) uranyl ion is considered to be the predominant bioavailable form of uranium responsible for toxicity, and to a lesser extent the hydrated (UO_2OH^{+}) uranyl ion (Markich *et al.* 2000). The speciation of U species in water is influenced predominately by pH and the number of complexing ions available, with the proportion of free and hydrated ions decreasing with increasing pH (Markich 2002).

Uranium is one of the primary contaminants of concern for the aquatic ecosystems downstream of the Ranger mine, and is present at various concentrations in ore and waste rock. Identified risks include mobilisation and leaching from contaminant sources under certain conditions, which may enter the surrounding aquatic environment through surface or groundwater after mine rehabilitation (Supervising Scientist 2018b). A site-specific GV of 2.8 µg/L has been derived for uranium (van Dam *et al*. 2017), and if this limit is exceeded strict data reporting and investigation is required from the mining company (Turner *et al.* 2015).

1.4 Freshwater mussels in the Alligator Rivers Region

The tropical freshwater mussel known as *Velesunio angasi* is distributed widely from north-western through to north-eastern Australia (Klunzinger *et al.* 2010) and is common in many permanent billabongs and creeks within the ARR, such as the Magela Creek system. One study estimated the mean density of mussels in a 50 m reach of the Magela Creek to be 7.1 mussels/m, with a total biomass (without shell) thought to exceed that of other invertebrates (Humphrey 1995). *Velesunio angasi* is an important part of the diet of the Aboriginal people living downstream of the Ranger uranium mine, and, due to its inherent capacity to accumulate some radionuclides, metals, and other contaminants, a program has been in place since 2000 to monitor radionuclide and metal concentrations in mussel tissues, and the surrounding water and sediment. The aim of the monitoring program is to inform management of the effects of mine waste waters to ensure that the mussels remain fit for human consumption (Supervising Scientist 2015). Part of a study by Allison and Simpson (1989) explored the dietary intake of mussels by Aboriginal people inhabiting the Alligator Rivers region. By counting discarded shells, conservative estimates of the number of mussels consumed were 60 per person throughout the dry season of 1980, and 288 throughout the dry season (May to September) of 1981. This information helped to estimate the concentrations of elements consumed from the mussels (which can bioaccumulate metals and radionuclides) and whether these remained within levels considered safe for human consumption. The study demonstrated the importance of mussels in the diet of Aboriginal people. However, the numbers of mussels consumed may have been under-estimated, as some potential eating sites may have been excluded, and consumption varied from year-to-year.

Extensive biological and ecological studies of *V. angasi* inhabiting permanent billabongs and braided creek channels in the ARR were undertaken by Humphrey and Simpson (1985). They noted that McMichael and Hiscock (1958) had previously reported the immense variation in shell shape amongst a single Hyriid species. A number of different and distinguishable growth forms were observed within the catchment of Magela Creek. For example, *V. angasi* shells from billabongs resembled those of *V. wilsonii*. Variability (or ecophenotypic variation) between creek and billabong samples was thought to be environmentally induced by factors such as temperature, water depth and flow rate, substrate type, trophic degree and size of the habitat, and water turbulence (Humphrey and Simpson 1985).

Two main shell forms of *V. angasi* recognised by Humphrey and Simpson (1985) were the billabong form and the creek form. The billabong form (Figure 1.5) was described as true *angasi* form, being expanded posteriorly with the dorsal margin abruptly truncated to form a pronounced wing. Wing development was less obvious in larger shells, and the periostracum (shell) was shiny. The creek form (Figure 1.6) was found in the braided, sandy creek channel which is exposed to rapid water flow during the wet season, but dries out into sandy creek beds and small pools of water during the dry season. These mussels inhabited root mats of aquatic or bankside vegetation, and roots of the *Pandanus*. They were described as an ecophenotypic variant of the billabong or *angasi* form, and were distinctly obese and winged, with a dull periostracum exhibiting fine growth lines (Humphrey and Simpson 1985).

Figure 1.5: The billabong shell form of *Velesunio angasi* from Sandy Billabong (Image: L. Kleinhenz).

Figure 1.6: The creek shell form of *Velesunio* sp. from Magela Creek. The periostracum on the beak has been abraded with age (Image: L. Kleinhenz).

Spawning and breeding for *V. angasi* may occur throughout the year in billabongs, however low water temperatures, turbidity, and changes in dissolved oxygen may limit reproduction rates. Reproduction in Magela Creek is confined to the wet season when the creek is flowing. During cessation of flow in the dry season, mussels hibernate by burrowing into sandy banks and the roots of Pandanus trees (Humphrey and Simpson 1985).

The transformation of glochidia (Figure 1.7) into juveniles (Figure 1.8) takes from four to fourteen days, and is dependent on the availability of host fish, as well as water temperature. Larval development can be completed in less than ten days during the wet season (Humphrey and Simpson 1985). Glochidia with hooked teeth (such as *V. angasi*) are reported to have generalist host-fish requirements Klunzinger *et al*. (2010). Humphrey and Simpson (1985) found no evidence of host fish specificity for the glochidia of *V. angasi*, and identified 19 fish species in the Magela Creek area that were fish hosts for *V. angasi.* Bottom-feeding fish species such as *Mogurnda mogurnda* were found to be the most successful hosts for glochidia.

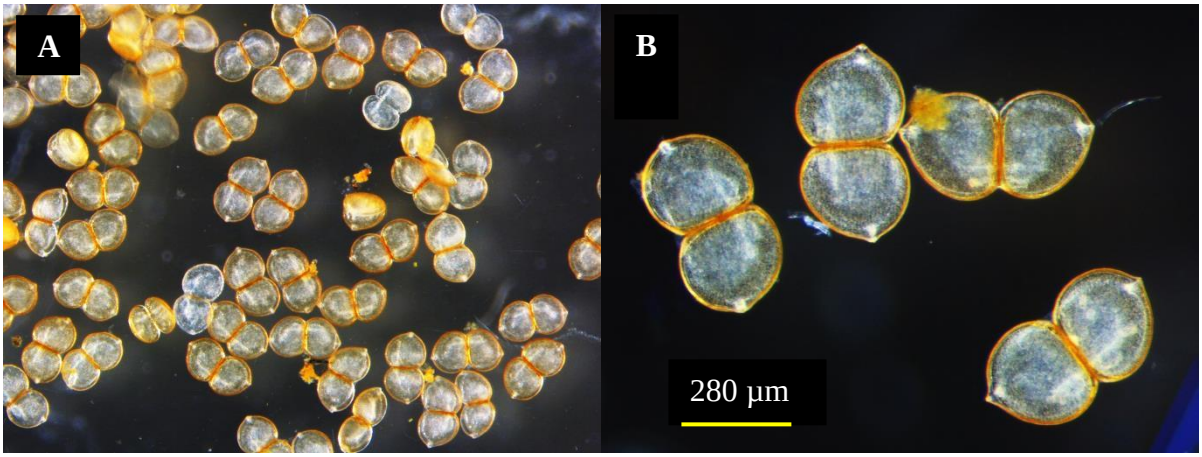

Figure 1.7: Glochidia of *Velesunio angasi*, less than 24-h after release from adult female mussel (A), and zoomed in to show the adductor muscle and teeth (B). Image: L. Kleinhenz

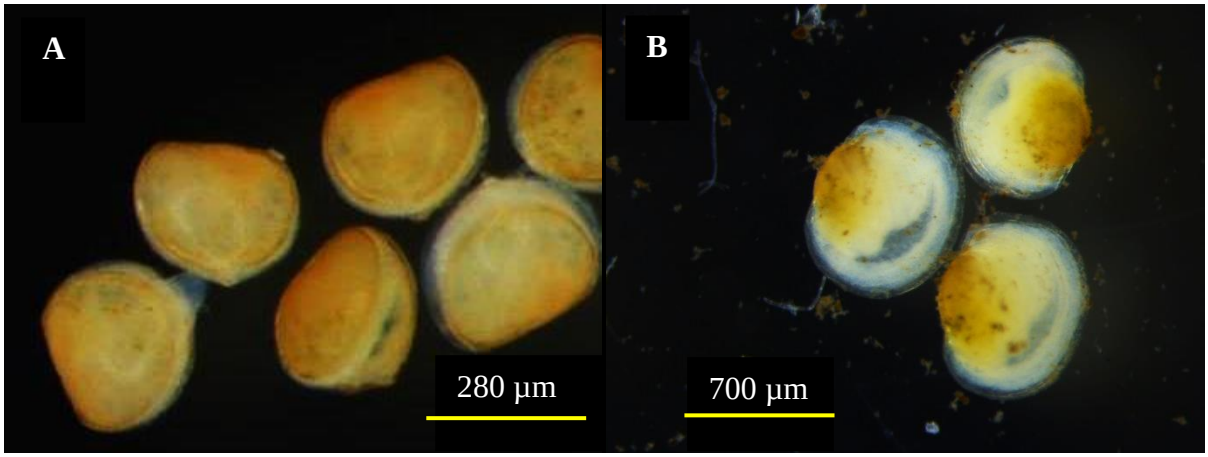

Figure 1.8: Juveniles of *Velesunio angasi,* (A) less than 24-h after excystment from host fish and (B) 14-d after excystment, showing growth and organ development.

Toxicity testing with *Velesunio* spp. glochidia has previously been undertaken to screen mine wastewaters using a novel toxicity test method (Humphrey 1987a), using reduced glochidial snapping rate as a sensitive indicator of toxic effects. A decrease in reproductive activity of adult *Velesunio angasi* was also shown following exposure to retention pond water released from the Ranger mine (Humphrey 1995), and the effects of U and Mn on adult *V. angasi* were assessed by Markich *et al*. (2000) using valve movement responses as the endpoint. However, toxicity testing on the early life stages of *Velesunio* spp. with ammonia, magnesium and uranium has not previously been conducted using standardised test methods.

The addition of the freshwater mussel to the suite of tropical test species will aid in quantifying the effects of internal dose on the mussels. Data from the present study may be utilised alongside that of other species to inform the derivation and updates of site-specific, water quality GVs for each contaminant. The data may also be used for future revisions of site-specific water quality GVs for these contaminants, as well as ensuring the protection of this important resource.

1.5 Thesis objectives

The hypothesis of this research was that the contaminants of potential concern from the Ranger mine may reduce the survival and growth of tropical freshwater mussels at environmentally relevant concentrations, and this can be quantified using standardised laboratory toxicity tests.

The objectives and research questions relevant to this study are:

1. To investigate the genetic diversity of freshwater mussels collected from various field sites throughout tropical Northern Territory, Australia, to determine if different species exist, and whether any genetic variability affects sensitivity to contaminants.

- *Do genetic variations exist within freshwater mussels found throughout the northern part of the Northern Territory?*

2. To adapt and optimise acute and chronic laboratory toxicity test protocols suitable for assessing the glochidia (larvae) and juveniles of tropical freshwater mussels.

- *What are the most suitable culturing methods for optimal Velesunio* spp. *survival and growth?*
- *Which measurement endpoints of larval and juvenile survival and development are most suitable for use in toxicity testing?*
- *Can a standard acute and/or chronic toxicity test be developed for Velesunio* spp.*?*

3. To use the optimised toxicity test protocols to assess the acute and chronic toxicity of contaminants of potential concern (ammonia, uranium, magnesium) from the Ranger uranium mine to the mussels.

- *How toxic are ammonia and other mining contaminants to the early life stages of Velesunio* spp.*?*
- *Are current site-specific water quality guideline values for key contaminants adequate for the protection of Velesunio* spp.*?*

1.6 Structure and outline of thesis

This thesis consists of 8 chapters, each contributing towards addressing one or more research aims of the study. Several chapters are presented as individual journal articles that are either published (Chapters 3, 4, and 5), or in preparation (Chapters 6 and 7). Supplementary information is included at the end of each chapter where applicable, and a combined reference list is provided at the end of the thesis.

Chapter 1 (this chapter) provides a background introduction and literature review.

Chapter 2 presents the findings of genetic analysis of tropical freshwater mussels from tropical northern territory.

Chapter 3 presents a published journal article describing the acute toxicity test method optimisation using water-only exposures, and assessment of ammonia toxicity to glochidia.

Chapter 4 presents a published journal article describing the chronic toxicity test method optimisation including the addition of fine silt, and an assessment of ammonia toxicity to juvenile mussels.

Chapter 5 presents a published journal article describing the acute and chronic toxicity of tropical freshwater mussels to magnesium.

Chapter 6 presents a journal article in preparation describing the acute toxicity of tropical freshwater mussels to uranium and copper.

Chapter 7 presents a journal article in preparation investigating the effects on water chemistry and juvenile mussel growth and survival in chronic uranium toxicity tests using pre-spiked and un-spiked sediments.

Chapter 8 concludes the thesis with a final discussion, summarising the major findings and limitations of the research, and providing suggestions for future research needs.

CHAPTER 2: Genetic identification of tropical freshwater mussel species for use in standardised toxicity testing.

Abstract

A key issue when developing standardised toxicity test protocols is to ensure the test species being used is accurately characterised. Genetic differences between species may influence their tolerance to environmental contaminants and many ecologically and culturally important species require appropriate protection from water quality perturbations. Cryptic species are common amongst freshwater mussels, but data are lacking on the population and taxonomic status of many species. In the present study, mitochondrial DNA analysis was used to confirm the identity of tropical freshwater mussels from Northern Australia that were to be used in standardised toxicity tests. Adult mussels were sampled from 13 sites in tropical northern Australia; 8 sites from within the Alligator Rivers Region (ARR), and 5 outside of the ARR. Total genomic DNA was extracted from mantle tissue from each mussel sample, and a 710-bp fragment of the mitochondrial COI gene was amplified for analysis. A phylogenetic tree of 200 sequences was constructed, indicating the presence of three known and three to four unknown or undescribed species. Billabong-sourced mussels matching *Velesunio angasi* were genetically different from creek-sourced mussels, the latter of which did not closely match any known sequences. The results of this study contribute to genetic knowledge of freshwater mussels in northern Australia, and can be used to clearly identify mussels used in future toxicity tests.

2.1 Introduction

When using ecotoxicological testing to evaluate the sensitivity of organisms to contaminants, accurate identification of the test species is important, because even small genetic differences between closely related species may result in variations in sensitivity (Sturmbauer *et al*. 1999; Monteiro *et al.* 2018). This can have implications when closely related species are used for environmental management purposes, because inaccurate assumptions about their tolerances can be made (Monteiro *et al*. 2018).

Freshwater mussels are notorious for morphological variability and phenotypic plasticity due to environmental conditions (Zieritz *et al*. 2018), including water velocity and water depth, sediment grain size, food availability, infestation by parasites, and food availability (Morais *et al*. 2013, and references therein). Traditional methods of taxonomy for freshwater mussels based on the morphology of larvae, shells, and soft parts (Baker *et al*. 2004) can be unreliable because of the intraspecific variation and interspecific convergence commonly observed in mussel shell morphology (Jeratthitikul *et al.* 2019).

Knowledge of the taxonomic status of mussel species is important in understanding their evolutionary history, forming reliable classifications, and for managing their conservation (Lopes-Lima *et al*. 2017). Molecular methods of identification are being used widely in North American research to help provide more reliable identification for Unionid mussels (e.g. Whelan *et al*. 2011), but not frequently in Australia where species diversity is lower (Baker *et al*. 2004). Mitochondrial DNA (mtDNA) analysis is a useful tool for studying species relationships. For example, phylogenetic positions and taxonomic status of freshwater mussel species that were previously hindered by taxonomic uncertainties have recently been resolved using mtDNA analysis in North America (Inoue *et al*. 2018) and China (Huang *et al.* 2019; Wu *et al.* 2019). In Australia, mtDNA analysis of freshwater mussel species has been used successfully along with morphological taxonomy to identify four cryptic *Velesunio* lineages as separate species (Baker *et al*. 2003, Baker *et al*. 2004), and was subsequently used along with allozymes to determine connectivity patterns of each of the four cryptic species (Hughes *et al*. 2004).

Global declines of freshwater mussel species are well-documented, particularly in North America where 70% of the ~300 known species are listed as endangered, threatened, or of special concern (Baker *et al*. 2004; Augspurger *et al*. 2007). In Australasia, seven of the 32 known species are listed as threatened (Walker *et al*. 2014). Major causes of global declines

include water pollution, sedimentation (Augspurger *et al*. 2007), over-exploitation, introduction of invasive species and climate change (Lopes-Lima *et al*. 2017).

Freshwater mussels are common in the waterways of tropical Northern Territory, Australia, including creeks and billabongs within the Alligator River Region, within which lies the world heritage listed Kakadu National Park and Ramsar listed wetlands (Sinclair *et al*. 2013). Mussels are important both ecologically and culturally, being part of the diet of Aboriginal communities living in the region, including downstream of the Ranger uranium mine. The biology and ecology of freshwater mussels in this region have previously been studied, and were originally identified as *Velesunio angasi*, based on morphological characteristics (Humphrey and Simpson 1985). Although several different shell forms had been identified in this early work, genetic analysis of mussel populations inhabiting the region had not been carried out prior to this study to confirm species identity.

2.2 Materials and methods

2.2.1 Field collections

Adult mussels were collected between October 2015 and May 2018 from 13 sites throughout the northern part of the Northern Territory (Table 2.1). Eight sites were situated within the Alligator Rivers Region (Figure 2.1), and five sites were situated in catchments outside of the Alligator Rivers Region. Fifteen mussels were collected from each site, with the exception of Katherine River, for which 15 mussels of each of the two observed phenotypes were collected. Mussels were transported live to the laboratory in 20-L aerated plastic drums containing approximately 15 L of water collected from the site, within 4-h of collection. In the laboratory, mussels were transferred into aerated 144-L glass tanks with a layer of sand, where they were held for up to two weeks until processing. An exception to this was the mussels collected from a spring on the Magela Floodplain, which were placed in a container of 70% ethanol directly after collection.

Table 2.1: Details of each mussel collection site. Sites 1-7 were within Kakadu National Park.

	Collection date	Site Code	Name	Catchment region	Latitude (S)	Longitude (E)
1	14/11/2015	MFP	Magela Flood plain	East Alligator River	12° 23’ 34.71”	132° 50’ 35.69”
2	22/10/2015	MB	Mudginberri Billabong	East Alligator River	12° 35’ 33.28’’	132° 52’ 33.68’’
3	03/03/2017	GC	Gulungul Creek	East Alligator River	12° 39’ 20.86’’	132° 52’ 42.43’’
4	03/12/2015	MC	Magela Creek	East Alligator River	12° 40’ 58.93’’	132° 56’ 23.30’’
5	16/11/2016	BB	Bowerbird Billabong	East Alligator River	12° 46’ 18.05’’	133° 02’ 22.98’’
6	04/05/2018	NC	Nourlangie Creek	South Alligator River	12° 51’ 22.01’’	132° 46’ 38.42’’
7	23/10/2015	SB	Sandy Billabong	South Alligator River	12° 54’ 09.38’’	132° 46’ 50.52’’
8	26/11/2015	SAR	South Alligator River	South Alligator River	13° 28’ 37.74’’	132° 27’ 42.07’’
9	10/12/2015	CC	Coomalie Creek	Adelaide River	13° 00’ 58.41’’	131° 07’ 19.35’’
10	10/1/2017	LB	Lake Bennett	Adelaide River	12° 57’ 33.25’’	131° 09’ 52.78’’
11	10/12/2015	MD	Manton Dam	Adelaide River	12° 51’ 41.36’’	131° 07’ 12.63’’
12	10/12/2015	RJ	Rum Jungle Lake	Finniss River	13° 02’ 35.56’’	130° 59’ 52.16’’
13	26/11/2015	KR	Katherine River	Daly River	14° 29’ 25.03’’	132° 15’ 05.30’’

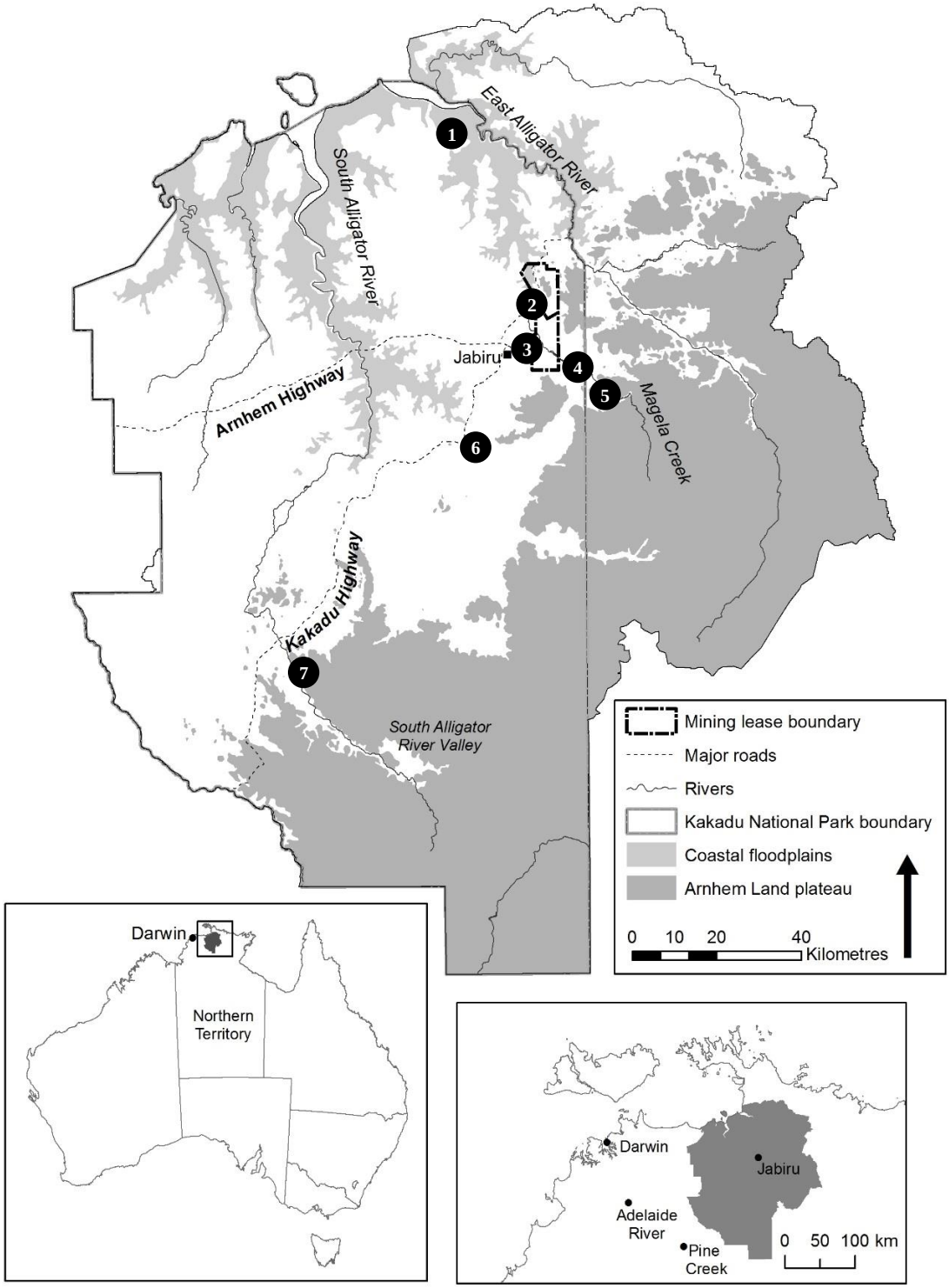

Figure 2.1: The Alligator Rivers Region of the Northern Territory, Australia, showing mussel collection sites. 1: Magela Creek floodplains, 2: Mudginberri Billabong, 3: Gulungul Creek, 4: Magela Creek, 5: Bowerbird Creek/Billabong 6: Sandy Billabong, 7: South Alligator River (map used with permission, Supervising Scientist 2015).

2.2.2 Sample preparation / preservation

Mussels were opened by inserting a scalpel blade between the valves and severing the anterior and posterior adductor muscles located on each end of the hinge. A small piece of mantle tissue was removed and preserved in 7-mL glass vials containing 99% ethanol. Lids were sealed with laboratory film (Parafilm "M") and the samples were then packaged in 1-L plastic jars packed with vermiculite to ensure no spillage occurred in transit. All samples were sent by road transport to Griffith University (Brisbane) for mitochondrial DNA analysis.

2.2.3 Mitochondrial DNA analysis

Total genomic DNA was extracted from mantle tissue using Qiagen DNeasy Blood & Tissue Kit (Qiagen). Extracted DNA was then diluted 1:50 with ultra-pure water, and amplified for the standard DNA barcoding region which represents a 710-bp fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene (Folmer *et al.* 1994). Ten microlitre reactions contained 2.0 µL PCR buffer (5x), 0.2 µL each LCO-1490 and HCO-2198 (10 mM), and 0.03 µL *Thermus aquaticus* DNA (MyTaq) polymerase (5 units/µL, Bioline). These were then subjected to the following PCR protocol: an initial hold of 95˚C for 5 mins, then 35 cycles of 95˚C for 60s, 40˚C for 60s, 72˚C for 90s, with a final extension at 72˚C for 7 mins. Amplified DNA was purified with enzymes Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher) as per manufacturer's instructions. Purified DNA was then sent to Macrogen Inc. (Korea) for sequencing.

2.2.4 Phylogenetic analysis

The resulting 194 COI sequences were edited and aligned using Geneious software (Geneious version 9.1.4 http://www.geneious.com, Kearse *et al*., 2012), and compared with 6 voucher sequences derived from Australian Museum specimens from Fawcett (2009, Table 2.2). Sequences were also compared to the National Center for Biotechnology Information (NCBI) nucleotide database using a blast search to identify any close matches. A phylogenetic tree incorporating all 200 sequences was generated under the maximum likelihood criterion with bootstrapping, using RAxML 8.2.10 via the CIPRES high performance computing server (Miller *et al*. 2010; Stamatakis *et al.* 2014).

Table 2.2: Voucher specimens used for comparison (Fawcett 2009).

Sample code	Location	Morphological ID
AM_VA2_1_1	Adelaide River, NT	*Velesunio angasi*
AM_VA2_3_1	Adelaide River, NT	*Velesunio angasi*
AM_VA4_1_1	Berry Creek, NT	*Velesunio angasi*
AM_H1_2_1	Katherine River, NT	*V. wilsonii*
AM_LR1_2_1	Ord River, WA	*Lortiella rugata*
AM_LR1_4_1	Ord River, WA	*Lortiella rugata*

2.3 Results

Mitochondrial DNA analysis produced 194 field-collected mussel sequences and six sequences derived from museum voucher specimens (Appendix A). Together the 200 sequences produced a 678-bp alignment without gaps, stop codons or frame shifts, indicating that the data lacked obvious contamination from nuclear pseudo-genes. Phylogenetic analysis produced a maximum likelihood tree with three distinct clades designated as Clade A, Clade B, and Clade C (Figure 2.2). Uncorrected percentage divergence between Clades A-B, A-C and B-C was 11.1, 11.0 and 10.1% respectively.

Three *Velesunio angasi* voucher sequences were placed within Clade A. Of these, two were within the shallow collapsed sub-clade ("*V. angasi*" group), and the third was outside this group. All mussels collected from Sandy Billabong (SB), Mudginberri Billabong (MB), South Alligator River (SAR), Lake Bennett (LB) and Manton Dam (MD) belonged exclusively to the *V. angasi* group within Clade A (Figure 2.2).

None of the voucher specimens matched sequences in Clade B, nor was there a close match between Clade B and publicly available COI sequences on the NCBI nucleotide database. Mussels from Gulungul Creek (GC), Magela Creek (MC), and Magela floodplain (MFP) belonged exclusively to the potentially undescribed *Velesunio* species in Clade B. Interestingly, mussels from Bowerbird Billabong (BB), Coomalie Creek (CC) and Nourlangie Creek (NC) were a mixture of two or three species, some matching with the *V. angasi* group in Clade A, and some matching with the potentially unknown species in Clade B (Figure 2.2).

The two species from Katherine River matched with *V. wilsonii* (code KR within Clade A, Figure 2.2) and *Lortiella* spp. (code KRL within Clade C) as expected. The *V. wilsonii* voucher sequence was placed at the base of Clade A, closest to and nested within Katherine River (KR) samples. A third sub-clade within Clade A contained a group collected exclusively from the Rum Jungle (RJ) site, which was 5.5% divergent from the "*V. angasi*" group (Figure 2.2) and did not match to any voucher sequences. The two *Lortiella rugata* voucher sequences were placed in Clade C and nested among samples identified as "Katherine River *Lortiella*" (KRL, Figure 2.2).

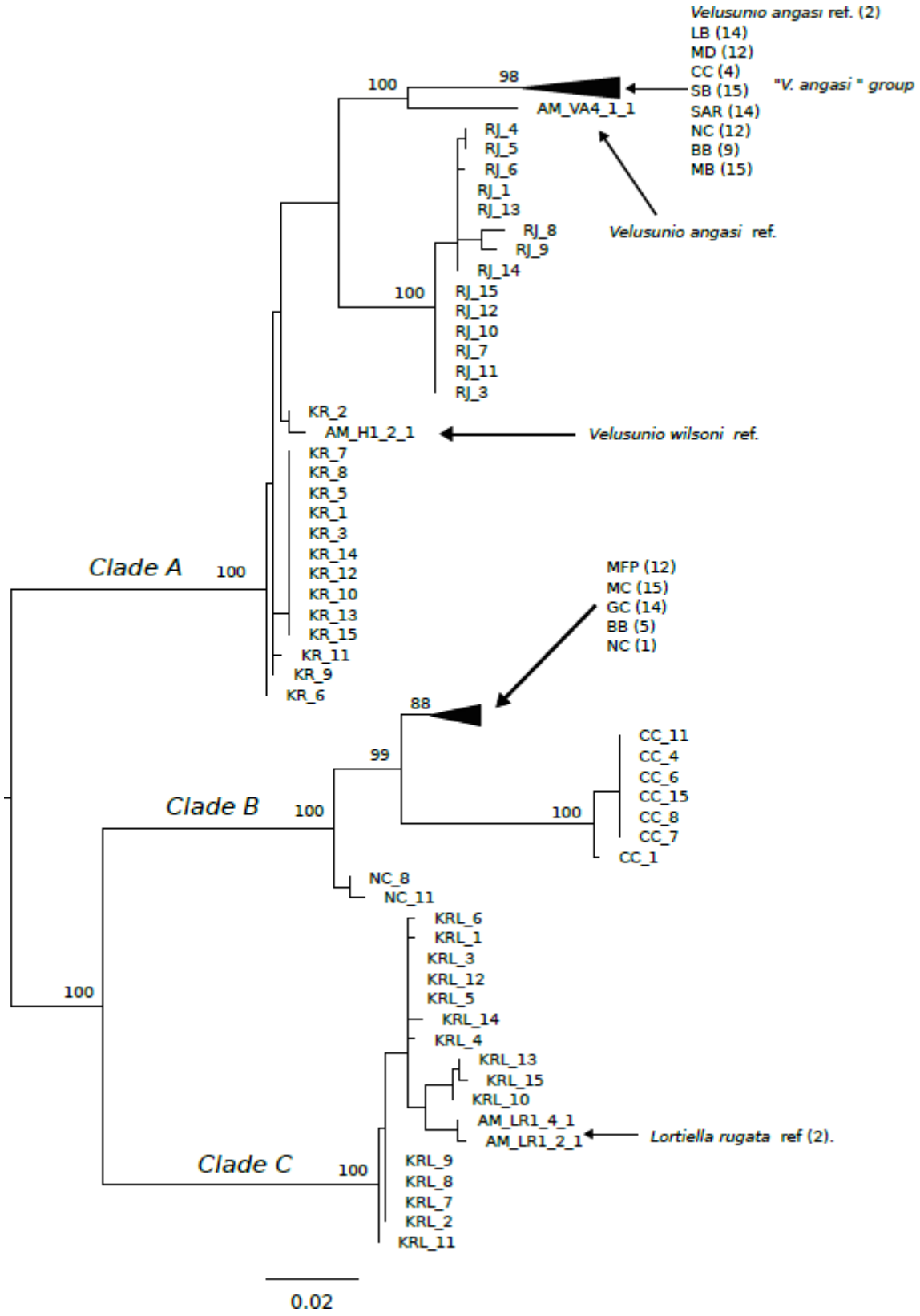

Figure 2.2: Phylogenetic tree showing three distinct clades (A, B and C). Uncorrected percentage divergence between clades A-B, A-C and B-C was 11.1%, 11.0% and 10.1% respectively. For ease of viewing, the tree features two collapsed large shallow sub-clades; one in clade A that consists of 97 tips, and one in clade B with 47 tips.

2.4 Discussion

Based on the genetic analysis, mussel populations with the potential for use in toxicity testing with contaminants of potential concern include *Velesunio* sp. from Magela Creek, Gulungul Creek, and Magela floodplain, and *Velesunio angasi* from Sandy Billabong, Mudginberri Billabong, South Alligator River, Lake Bennett, and Manton Dam. These populations are representative of the mussel species located within the ARR, and relevant to those that may be exposed to contaminants from the Ranger mine. Therefore, populations that did not represent species found in the ARR (RJ, KR, and KR-L) would not be considered relevant test species. Mussel populations containing two or more species occurring sympatrically (BB, NC, CC) would not be suitable if they could not be clearly identified. Field site locations and ease of collection are also important factors to consider. Populations that are easier to access (MC, GC, SB, MD, and LB) would be preferred over those more difficult to access (MFP, SAR, and MB).

The results of the mitochondrial analysis revealed three distinct clades among the 13 sampled sites. Of the three groups within Clade A, the *V. angasi* group (matching to *Velesunio angasi* voucher specimens) comprised of mussels from eight different populations representative of lentic water bodies exclusively (SB, MB, LB, and MD), or water bodies with both lentic and lotic characteristics (SAR, CC, NC, BB).

The groups within Clade B did not match any voucher specimens or known sequences, and, therefore, may represent one (or two) undescribed taxa, or alternatively, represent species not previously sequenced. Within Clade B, the 47 sequences in the collapsed group were tentatively referred to as *Velesunio* spp., on the assumption that they belonged to the same genus as *Velesunio angasi.* However, it is possible that they belong to a different genus, being as similarly divergent from other *Velesunio* species in Clade A as from the *Lortiella* species in Clade C. These 47 sequences represented mussels collected from five different sites. Three of these sites were lotic water bodies exclusively represented within this unknown mussel group: Magela floodplain (MFP), Magela Creek (MC), and Gulungul Creek (GC), while some sequences from the other two sites, Nourlangie Creek (NC) and Bowerbird Billabong (BB), which were representative of lotic and lentic water bodies, occurred sympatrically with the *V. angasi* group in Clade A. Sympatric occurrences of the lentic *V. angasi* and the undescribed lotic species within the Alligator Rivers Region appear to have occurred at sites where mussels have been collected close to billabongs, namely Bowerbird Creek and Nourlangie Creek. It is possible that the *V. angasi* found at these sites originated from the billabongs and were likely washed into the creek during the wet season.

In addition to the 47 sequences collapsed in Clade B, one sub-clade could represent a distinct, undescribed taxon – that is, the well-supported group within Clade B comprised of samples collected at the Coomalie Creek (CC) site, which is ~5% divergent from the collapsed sub-clade in Clade B (Figure 2.2). Clade A also contained a well-supported sub-clade that could represent an undescribed taxon, that is, the group collected exclusively from the Rum Jungle (RJ) site, which was 5.5% divergent from the "*V. angasi*" group (Figure 2.2). The results of these genetic analyses would be useful in future taxonomic studies but would require further investigation for definitive taxonomic resolution of the undescribed species. Such studies would involve further sampling and genetic analysis of the targeted species from the creek systems of the ARR, as well as morphological descriptions of live and preserved glochidia, juveniles, and adult mussels.

2.5 Conclusion

Mitochondrial DNA analysis was effective in revealing the presence of three clades among the 13 sampled sites. One group containing lentic species from eight sites was identified as *Velesunio angasi,* and two lotic species from Katherine River matched to known sequences: *Velesunio wilsonii*, and *Lortiella rugata.* One lotic species and six lentic species did not match any known sequences and may represent undescribed species. Further genetic study of the mussels in this region would be valuable in providing a comprehensive understanding of their taxonomic status and thus aid in their conservation. The results of this study will contribute to genetic knowledge of freshwater mussels in northern Australia, and will be used to inform mussel toxicity testing programs.

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CHAPTER 3: Acute ammonia toxicity to the larvae (glochidia) of the tropical Australian freshwater mussel *Velesunio* spp. using a modified toxicity test protocol

Abstract

Ammonia is recognised as a major pollutant worldwide, originating from natural and anthropogenic sources. Studies have reported that freshwater mussels are amongst the most sensitive taxa to ammonia, but few data are available on ammonia toxicity for the early life stages of freshwater mussels from tropical regions. We report on the modification of a 24-h acute toxicity test protocol for tropical freshwater mussels and application of the test using ammonia. *Velesunio* spp. from 3 different sites were used to assess the toxicity of ammonia at a targeted pH of 6.0 and a water temperature of 27.5°C, which were the average annual values for some slightly to moderately acidic, soft water (3-6 mg/L as $CaCO_3$) creeks of tropical northern Australia. The valve closure responses of mussel glochidia (larvae) to a sodium chloride solution were used to measure the survival endpoint. Acute toxicity estimates indicate that tropical *Velesunio* spp. were highly sensitive to ammonia, with 24-h exposures to ammonium sulfate generating median lethal concentration estimates ranging from 6.8 to 14.2 mg/L total ammonia nitrogen, which, when adjusted to pH 7 and 20°C, were among the highest sensitivities yet reported for any freshwater mussel species, and among the highest in sensitivity for any tropical taxon. These toxicity estimates can contribute to the derivation or refinement of ammonia guideline values for freshwater ecosystems globally.

Keywords: Ecotoxicology; Aquatic invertebrates; Mining; *Velesunio angasi*; Acute toxicity; Water quality guideline values

3.1 Introduction

The contamination of freshwater environments by ammonia, from both natural and anthropogenic sources, is a significant global problem, and thus the harmful effects of this toxicant on aquatic organisms have been extensively studied (Augspurger *et al*. 2003; Wang and Leung 2015). Ammonia is used for numerous industrial applications, including the synthesis of many organic and inorganic chemicals, in the manufacture or direct application of fertilisers, and in the mining industry for the extraction of metals (USEPA 2013). Some mine sites must address the potential for off-site ammonia contamination, which may occur through the leaching of contaminated waters into groundwater or potential mine water spillage into surface waters (Sinclair *et al*. 2013; van Dam *et al*. 2002).

In the water column, the ammonium ion (NH_4^+) and un-ionised ammonia (NH_3) occur in equilibrium. The ratio of these 2 forms depends primarily on the pH and temperature of ambient waters, with a rise in either of these variables causing the concentration of NH_3 to increase and the concentration of NH_4^+ to decrease (Emerson *et al*. 1975). Because its neutral charge allows easier diffusion across cell membranes (USEPA 1999), NH3 is considered more toxic than NH_4^+ to aquatic organisms. In general, overall toxicity is greater at higher pH where more NH_3 is present. Total Ammonia Nitrogen (TAN), the sum of the NH_3 and NH_4^+ concentrations, is used as a measure of ammonia in water samples (ANZECC/ARMCANZ 2000; USEPA 2013).

Most toxicity data used for deriving water quality guideline values have been generated using temperate species from North America and Europe. Many tropical water quality guideline values are extrapolated from temperate data due to the lack of tropical data available, with the assumption that sensitivity to toxicants is similar (Kwok *et al.* 2007). However, the use of temperate data presents significant uncertainties regarding the adequacy of the derived guideline values for protection of tropical ecosystems (Wang and Leung 2015). Kwok *et al*. (2007) observed that the quality of tropical toxicity data was generally lower than that for temperate data, because the latter data are based upon a greater number of standardised test organisms and test procedures. They recommended that safety factors of between 1 and 10 be applied (depending on the desired protection level) when temperate datasets are used to predict toxicity to tropical species, and suggested further validation of these safety factors by conducting toxicity tests with untested chemicals on both tropical and temperate species. In addition, they identified a major toxicity data gap for molluscs in particular in tropical regions (Kwok *et al.* 2007). Acute tropical toxicity data for ammonia

using valid endpoints for guideline value derivations (ANZECC/ARMCANZ 2000), had been limited to only 2 taxonomic groups (amphibians and fish), until a recent study added acute toxicity data for 10 tropical species covering 5 taxonomic groups (algae, crustaceans, fish, molluscs, and one insect; Wang and Leung 2015). A probabilistic risk assessment of these collective data concluded that ammonia was likely to be more toxic to tropical than temperate organisms. An extrapolation factor of 4 was recommended when temperate ammonia toxicity data are used to protect tropical freshwater ecosystems (Wang and Leung 2015). Acute ammonia data for tropical Australian species are limited to a published study that provided acute data for juvenile barramundi (*Lates calcarifer*; Økelsrud and Pearson 2007), and a Master's thesis that provided acute data for juvenile *L. calcarifer*, eastern rainbowfish (*Melanotaenia splendida splendida*), and a freshwater shrimp (*Caridina nilotica*; Økelsrud 2004). The barramundi were the most sensitive to ammonia, with acute toxicity values comparable to highly sensitive, temperate salmonid species (Økelsrud and Pearson 2007; Økelsrud 2004). The eastern rainbowfish sensitivity was comparable to that of a range of temperate non-salmonids, whereas sensitivity of the tropical shrimp was greater than that of rainbowfish and comparable to that of other temperate shrimps and invertebrates (Økelsrud 2004).

The early life stages of temperate freshwater mussels (glochidia and newly-transformed juvenile mussels) were found to be highly sensitive to acute ammonia exposures when compared with the most sensitive species from other invertebrate, fish or amphibian groups (Augspurger 2003). Hence, it was recommended that freshwater mussels be included in the derivation of guideline values to ensure adequate protection of freshwater environments (Augspurger 2003; Wang *et al.* 2007a).

The reproductive cycle of unionoid freshwater mussels is complex. Sexes are separate, with larvae (glochidia) developing in the marsupial portions of the gills of adult females. At maturity, glochidia are released into ambient waters as obligate parasites, requiring attachment to gills or fins of a host fish to continue development (Kat 1984). Freshwater mussels of the genus *Velesunio* (Hyriidae) are found throughout large parts of Australia, with 3 or more species common in the billabongs, streams and rivers of tropical northern Australia. In the Alligator Rivers Region (NT, Australia), the biology and ecology of 2 species of *Velesunio* have been studied extensively by Humphrey and Simpson (1985), these species constituting an important part of the diet of local Aboriginal communities.

Uranium mining is conducted in the Alligator Rivers Region, and a toxicity testing program using local organisms has been undertaken with mining related contaminants,

including ammonia, to provide confidence that derived guideline values are adequately protective of the receiving water environment (van Dam *et al*. 2002). *Velesunio* spp. in the Alligator Rivers Region have been used for biomonitoring purposes (Humphrey *et al.* 2002), and radionuclide and metal concentrations in adult mussel tissues are measured annually to determine whether they remain below acceptable levels for human consumption (Bollhofer *et al*. 2011). In the past, *Velesunio* spp. glochidia have been used for pre-release screening of mine waste-waters (Humphrey 1987a; Holdway *et al*. 1987) using a novel toxicity test method developed by Humphrey (1987a). The method used the endpoints of valve closure ('snapping') rate and survival of glochidia over 96 h. Although snapping rate response was a sensitive indicator of toxicity, as a behavioural endpoint its ecological relevance was not established. The test method was also deemed overly time consuming for routine test application.

In more recent years, standardised toxicity testing protocols for freshwater mussels have been developed internationally in response to growing concerns about mussel species declines (ASTM 2006; Augspurger *et al.* 2007). These protocols have led to an increase in published mussel toxicity data for many contaminants (Bringolf *et al.* 2007; March *et al*. 2007; Wang *et al*. 2007a) including recent acute data for metals using 6 temperate Australian hyriid species (Markich 2017). Acute ammonia toxicity to temperate freshwater mussel species has been assessed (Wang *et al*. 2007a; Clearwater *et al*. 2014), but there are currently no acute data for tropical freshwater mussel species.

Velesunio spp. fulfil several criteria for selection of an appropriate test species for deriving guideline values, as recommended in ANZECC/ARMCANZ (2000) and USEPA (2013), that is, regional relevance and importance, and early life stage sensitivity to contaminants (Humphrey *et al.* 1989). Several refinements to the acute toxicity test protocol recommended by the ASTM (2006) are required when using *Velesunio* spp. for toxicity testing, due to their high ecological value, physiology, and behaviour, and the difficulty in collecting adult mussels. These include the method of acquiring glochidia from adult mussels, and the use of both smaller replicate numbers of glochidia, and smaller test volumes.

The main objectives of this study were to: 1) refine a standardised acute toxicity test protocol for freshwater mussel glochidia that can be used to assess a range of contaminants in conditions relevant to the tropics, and 2) use this test protocol to assess the toxicity of ammonia to tropical freshwater mussel glochidia. This information may contribute to refinement of site-specific, national and international water quality guideline values for ammonia and the associated environmental regulation of ammonia.

3.2 Materials and methods

The toxicity test protocol in this study was refined from the ASTM International Standard (ASTM E2455-06 Standard guide for conducting laboratory toxicity tests with freshwater mussels; ASTM 2006).

3.2.1 General laboratory procedures

All glass and plastic ware used in experiments were prepared by soaking in a 5% (v/v) nitric acid (HNO_3) bath for 24 h, and then triple rinsing in deionised water, followed by washing and rinsing in a laboratory dishwasher (Miele) using phosphate-free detergent (Dr. Weigert, neodisher® Laboclean) and deionised water.

Natural Magela Creek water was used as the control treatment and the diluent in all tests. Magela Creek water is typically very soft (Klessa 2000); the average physicochemical and major ion concentrations of the waters used in toxicity tests are shown in Table 3.1. Water collections were made monthly in 20-L acid-washed plastic containers from a permanent water body upstream of Ranger mine (Bowerbird Billabong, Magela Creek, NT, Australia; latitude 12°46’15’’, longitude 133°02’20’’); the waters thus collected were subsequently filtered in the laboratory (3 µm; Sartopure PP2 MidiCap filter, Sartorius Stedim) and then stored at 4°C until use.

Table 3.1: Average physico-chemical composition of 0.45 µm filtered Magela Creek water (MCW) used in definitive ammonia tests (n = 7) [a]

Physico-chemical variable	Analyte	MCW	Detection limit
pH	-	6.1 ± 0.0	-
Conductivity (µS/cm)	-	19.4 ± 4.5	-
Dissolved oxygen (%)	-	99.2 ± 5.3	-
Alkalinity (mg/L as $CaCO_3$)	-	2.8 ± 1.9	-
Hardness (mg/L as $CaCO_3$)	-	3.0 ± 1.3	-
DOC (mg/L)	-	2.7 ± 1.2	-
	Al (µg/L)	28.9 ± 30.7	0.1
	Cd (µg/L)	<0.02 ± 0.0	0.02
	Co (µg/L)	0.1 ± 0.0	0.01
	Cr (µg/L)	0.2 ± 0.1	0.1
	Cu (µg/L)	0.1 ± 0.1	0.01
	Fe (µg/L)	64.7 ± 11.2	1
	Mn (µg/L)	3.0 ± 2.7	0.01
	Ni (µg/L)	0.2 ± 0.1	0.01
	Pb (µg/L)	0.1 ± 0.1	0.01
	Se (µg/L)	<0.2 ± 0.0	0.2
	U (µg/L)	0.0 ± 0.0	0.001
	Zn (µg/L)	0.7 ± 0.3	0.1
	Ca (mg/L)	0.2 ± 0.1	0.1
	Mg (mg/L)	0.6 ± 0.3	0.1
	Na (mg/ L)	2.1 ± 1.0	0.1
	Sulfate, SO_4 (mg/L)	93.2 ± 8.5	0.5

[a] Values represent the mean ± standard deviation from control samples taken from each test.

3.2.2 Field collection of *Velesunio* spp.

The glochidia used throughout the present study were obtained from adult female mussels collected from various creeks and billabongs within the Alligator Rivers Region (Figure 3.1) and 2 locations outside of the Alligator Rivers Region: Lake Bennett, a semi-urbanised artificial lake situated 80 km south of Darwin (NT, Australia; latitude 12°57'39'', longitude 131°09'59''); and Manton Dam (NT, Australia), an historical water-supply now used for recreational purposes (latitude 12°51'42'', longitude 131°07'13'').

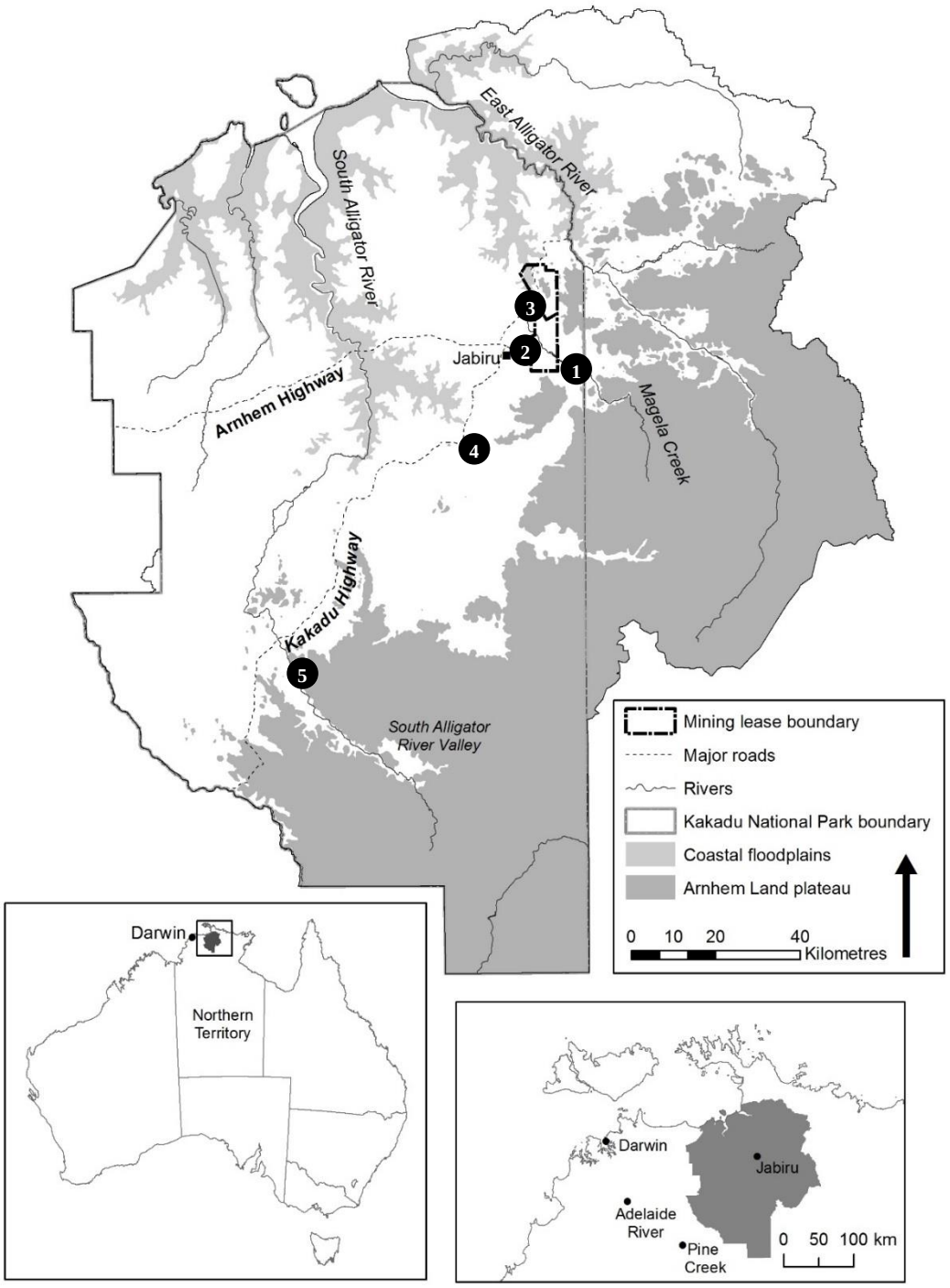

Figure 3.1: The Alligator Rivers Region of the Northern Territory, Australia, showing mussel collection sites. 1: Magela Creek, 2: Gulungul Creek, 3: Mudginberri Billabong, 4: Sandy Billabong, 5: South Alligator River (map used with permission, Supervising Scientist 2015).

All populations were previously believed to be just one species, *Velesunio angasi* (McMichael and Hiscock 1958; Humphrey and Simpson 1985). However, recent molecular genetics analysis concurrent with the present study (Environmental Research Institute of the Supervising Scientist, Australian Department of the Environment and Energy, Canberra, ACT, Australia and Griffith University, Brisbane, Qld, Australia, unpublished data) indicate the presence of another undescribed *Velesunio* species, termed *Velesunio* sp. throughout the present study. Mussels used in the present study represented both species, *V. angasi* occurring in permanent (often lentic) waters, and *Velesunio* sp. occurring in seasonally flowing stream channels that may dry out completely in the dry season. During test refinement (i.e., outside of exposure to toxicants), it was assumed that control performance and endpoint responses were similar across populations and species. For definitive ammonia toxicity tests, the species-specific responses were examined. Field collected mussels were transported back to the laboratory at ambient temperature (~28-32°C) within 4 h of collection, in plastic aerated 20-L drums with lids, filled to ~15 L with collected water from the site. To minimise early release of glochidia during transport, the bottom and sides of the drums were lined with plastic mesh to help cushion and buffer excessive vibration and water movement.

3.2.3 Glochidia isolation

On arrival at the laboratory, adult mussels were prepared for glochidia release by placing each one in individual round, clear-polypropylene containers (~10 cm diameter) with Magela Creek water to a depth of approximately 2 cm, so that the top of each mussel shell was slightly exposed; the containers were then covered and placed overnight in a constant temperature incubator (~27.5 °C). The reduced water volume induced a stress response in gravid female mussels, causing them to expel glochidia from the inner gills, without harm to the mussels. Glochidia were usually released after the first night, and observations were taken of the number of gravid females and the developmental stage of glochidia or eggs and embryos released (Supplementary Data, Table S3.1). All glochidia used in definitive ammonia toxicity tests had been released from female mussels within 24 h of the start of the test, as recommended in ASTM (2006).

3.2.4 Glochidia viability and selection

For glochidia to be acceptable for testing, the recommended viability of glochidia obtained from each mussel must be ≥80% and preferably ≥90% (ASTM 2006). An effective method of assessing glochidia viability for toxicity testing is the observation of valve closure in response to a salt (NaCl) stimulus, as described in Wang *et al.* (2007a).

In the present study, due to the small replicate numbers of glochidia, two sequential viability screening procedures were adopted to ensure maximum glochidia viability at test commencement: 1) an initial viability test on the total mass of glochidia arising from each mussel, and 2) selection of individual glochidia for tests, from a pooled sample deemed viable from the first screening. The double-screening procedure ensured selection of the healthiest and most vigorous glochidia for definitive testing. These procedures are described as follows:

First, percentage viability was assessed on a subsample of approximately 50 to 100 glochidia from each adult female by exposing the subsample to a 240 g/L salt solution, and observing the valve closure response after 1 min using the following formula adapted from ASTM (2006): % survival (viability) = 100 × (number closed after NaCl added - number closed before NaCl added) / (total number open and closed after NaCl added).

Second, the remaining glochidia (unexposed to NaCl) from adult females that had achieved ≥80% viability were pooled using a plastic 2-mL pipette, and then allowed to acclimatise to the test water (Magela creek water) and test temperature for approximately 3 h prior to testing, during a series of three 50% water changes. Selection of glochidia for testing was refined throughout the study. Initially, in the first 2 developmental tests, open glochidia from the pooled sample were selected indiscriminately and used in toxicity tests. In later developmental tests, only glochidia that snapped spontaneously when observed under a stereo microscope (Leica MZ8) were selected individually for tests. In later developmental tests and all definitive toxicity tests, open glochidia were selected individually under the microscope by their physical appearance (including snapping behaviour) when selection by snapping alone proved too time-consuming. Visible adductor muscles, well-developed teeth, open and spontaneously snapping valves, and translucent appearance were used as indicators of health and viability of mature glochidia (Humphrey and Simpson 1985; Supplementary Data, Figure S3.1). Selected glochidia were drawn up individually with a 1-mm glass pipette and transferred directly into test vessels (usually only 10-20 glochidia per replicate). Test vessels

were randomly arranged on Perspex trays and placed in a constant temperature incubator at 27.5°C (Labec).

3.2.5 Test protocol refinement

The test protocol was refined in a series of experiments by modifying various test conditions recommended in ASTM (2006). Preliminary trials focused on optimising pH control, test volume and test vessels (9-cm plastic Petri dishes with 30 mL of test solution vs 12-well plates submerged in a 700-mL rectangular plastic polypropylene container filled with 500 mL of test solution; test set-up shown in Supplementary Data, Figures S3.2 and S3.3), number of glochidia/replicate (10 vs 20/replicate), age of glochidia at test initiation (<20 h, 24 h, 26–42 h and 60–72 h after release from adult females), and test duration. One-way analyses of variance (ANOVAs; SigmaPlot Ver 13.0) were performed where necessary to determine any statistical differences among treatments.

Two different methods were trialled when pH control of test solutions was optimised. Strict control of pH was deemed necessary because ammonia toxicity increases approximately 10-fold for each unit pH increase (USEPA 2013). The first method used sealed air-tight chambers charged with elevated atmospheric CO_2 gas. Depending on the initial pH of Magela Creek water, 80 to 100 mL of CO_2 gas (0.29–0.36%) was injected into each chamber to control and maintain pH close to the site-specific level of 6.0 (Elphick *et al.* 2005). The second method used buffers to control pH. Two buffers, N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid (HEPES; Sigma-Aldrich) and 4-Morpholineethanesulfonic acid (MES; Sigma-Aldrich) were tested on glochidia in Magela Creek water to assess both control survival and effects on water quality.

Following refinement of glochidia selection, the survival of glochidia in Magela Creek water was trialled in 2 tests over 120-h to confirm the most suitable test duration. Natural survival rates of glochidia in the water column between release from female mussels and attachment to a host fish are relatively short (Wang *et al.* 2007a). Previous unpublished data on natural survival of *Velesunio* spp. glochidia (C.L. Humphrey, 1986, Humphrey, 1987) were compared to the current survival data. Unpublished data (C.L. Humphrey, 1986, Humphrey, 1987) were also assessed to compare the sensitivity of 2 different endpoints, survival and snap rate of *Velesunio* spp. glochidia that were exposed to 2 different Ranger Mine retention pond (RP) waters, RP2 and RP4. The composition of retention pond waters varied, with RP2 water being notably higher in uranium (Holdway *et al*. 1987).

3.2.6 Toxicity tests with ammonia

The final test protocol is summarised in Table 3.2. Seven definitive 24-h ammonia tests were conducted using the final test protocol to calculate toxicity estimates: 2 tests with *Velesunio* sp. collected from the sandy banks of Magela Creek, upstream of the Ranger Uranium Mine (site 1, Figure 3.1), where populations are not generally influenced by mine waste-water discharges, 3 tests with *V. angasi* collected from Sandy Billabong (site 4, Figure 3.1) and 2 tests with *V. angasi* collected from Lake Bennett. Static tests were conducted with glochidia <24 h old (after release from adult mussel), using 9-cm plastic Petri dishes containing 30 mL of test water. Ten glochidia were used in triplicate treatments (i.e., 30 glochidia/treatment).

Table 3.2: Details of the final 24-h acute toxicity test protocol

Test organism	
Species	*Velesunio* spp.
Life stage	Glochidia <24 h old post-release from adult female
Toxicity test description	
Type	Static
Test duration	24 h
Test vessels	9-cm plastic petri dishes with lids
Test solution volume	30 mL
Dilution water	Natural Magela Creek Water
# of test organisms per vessel	10
# of replicates per concentration	3
Assessment endpoint	Survival as measured by valve closure response after exposure to NaCl solution
Quality Control	
Test acceptability	Mean control survival ≥90%
Temperature	27.5°C ± 1°C
pH	6.0 ± 0.3
pH control	1 mM HEPES buffer adjusted to pH 6.0
Electrical conductivity	Change between start and end values ≤10%
Dissolved oxygen	80-120% saturation
Photoperiod	12:12-h (light: dark)

HEPES = N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid.

3.2.7 Preparation of test solutions

Stock solutions were prepared by adding analytical grade ammonium sulfate (NH_4SO_4; Sigma-Aldrich), to ultra-pure water (18MΩ/cm, Milli-Q, Millipore). Whilst international researchers have used ammonium chloride for mussel toxicity tests (Newton and Bartsch 2007; Wang *et al.* 2007a) the sulfate ion was used throughout this study for consistency with previous site-specific research, due to its environmental relevance (van Dam *et al.* 2010; Mooney *et al.* 2016). Test solutions were prepared by mixing the stock solution with Magela Creek water to make nominal concentrations of 1 to 36 mg/L total Ammonia Nitrogen (TAN). Test solutions were acclimated to test water temperature before test commencement. To control pH within ±0.3 units, 1 mM HEPES buffer was added to each test solution at the start of the test, and the pH of each test solution was adjusted with a small amount of 5% H_2SO_4 or NaOH until the starting pH was approximately 6.0.

3.2.8 Water quality

Water quality parameters (electrical conductivity, pH, and dissolved oxygen) were measured on pooled samples from the replicates of each treatment at the start and end of toxicity tests. The temperature of the incubator was monitored throughout each test using remote logging software (Testo Saveris™). Tests were considered valid if, at the end of the test, ≥90% of control glochidia survived, changes in electrical conductivity remained within 10% of the values measured at the start of the test, changes in pH did not exceed 0.3 units, dissolved oxygen levels remained above 80%, and the temperature of the incubator did not fluctuate more than 1º C.

3.2.9 Chemical analysis

Subsamples of newly collected Magela Creek water (unfiltered and 0.45 µm filtered) were analysed for dissolved organic carbon and alkalinity. Blank samples and diluent water samples from each test treatment were subsampled, filtered, acidified to 1% HNO_3 and sent for chemical analysis to a laboratory accedited by the Australian National Association of Testing Authorities (Envirolab, Chatswood, NSW, Australia) to determine the presence of metals and major ions (Al, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Na, Ni, Pb, Se, SO4, U and Zn) using inductively coupled plasma-atomic emission spectroscopy and inductively coupled plasma-mass spectrometry to detect any confounding contamination. Ammonia toxicity test treatments were prepared and diluted concentrations were tested in house to check against nominal concentrations by using an ammonia test kit (Palintest, 0 – 1.0 mg/L N detection

range), followed by spectrophotometry (Shimadzu UV-2550, wavelength 640 nm) analysis against a standard curve. Measured concentrations were calculated by averaging the spectrophotometer readings from the water samples taken at the start and finish of each test. The desirable maximum change between measured ammonia concentrations at the start and end of each test was 10%.

3.2.10 Statistical analyses

At the completion of each 24-h ammonia toxicity test, responsive and non-responsive glochidia were counted using the viability test (described in *Glochidia viability and selection*) to measure survival. Measured concentrations were used when lethal concentrations were calculated. Toxicity estimates for each toxicity test were calculated from concentration-response curves generated using non-linear regression models (CETIS™ Ver 1.9.0.9). The best-fit model was determined by the highest regression coefficient (r^2) of a limited suite of models (3- and 4-parameter logistic or sigmoidal). Ammonia concentrations at which there was a 50% reduction in survival (LC50; i.e. selected toxicity estimates and their 95% confidence limits), were calculated using the model equations.

3.2.11 Data comparisons

When guideline values are derived for ammonia, different models are used to adjust toxicity values to a given pH and temperature, and are either reported in TAN (USEPA 2013; ANZECC/ARMCANZ 2000) or the NH_3 proportion (CCME 2010). For the purpose of making comparisons with other published data, the toxicity values for the present study were reported in TAN by adjusting the geometric means of LC50s for tests from each of the 3 sites to pH 7 and 20 °C and pH 8 and 20 °C using the algorithms described in Emerson *et al*. (1975) and USEPA (1999). The adjusted toxicity estimates for ammonia (in mg/L TAN) were compared with available temperate freshwater mussel species data, and with ammonia data for tropical species from other taxa.

3.3 Results

3.3.1 Quality assurance / quality control

For all definitive ammonia tests, key mean physicochemical variables representing control and treatment waters at 0 and 24 h remained within acceptability criteria: pH 6.1 ± 0.04, dissolved oxygen 100.2 ± 5.7%, and temperature 27.5 ± 0.1°C (Table 3.3). As expected, the electrical conductivity increased with increasing ammonium sulfate concentration. The mean electrical conductivity of control treatments for the 7 tests was 19 µS/cm and the mean electrical conductivity of ammonia treatments (0.9–37.4 mg/L TAN) ranged from 26 to 413 µS/cm. However, this value did not change significantly within concentrations for the test duration, remaining within 2.5 ± 0.9% of the electrical conductivity at test commencement (Table 3.3). Measured ammonia concentrations generally remained within 10% between the start and the end of tests (average net increase of 1.2%; range of -13.2%–12.8%; Supplementary Data, Table S3.2). Analysis of blank and diluent samples showed no contamination of the measured variables in diluent water used in preparation of the stock solution, and Magela Creek water showed a stable composition throughout each test (Table 3.1 and Supplementary Data, Table, S3.3–S3.5), except for test 7, which showed an elevated Al concentration of 96 µg/L in Magela Creek water (Supplementary Data, Table S3.5). Due to insufficient data, it is unknown if Al would have had any significant toxic effects on glochidia at this concentration. However, a recent study on the toxicity of Al to juveniles of the temperate unionid mussel *Lampsilis siliquioida* reported a low acute sensitivity with median effect concentration of >6200 µg/L (Wang *et al.* 2018a).

The initial viability of all subsamples of glochidia representing the total mass from each mussel used in ammonia tests averaged 90.3 ± 3.7%, ranging between 85.9 and 96.0% (data not shown). Although this was below the desired 90% in some cases, the second viability screening ensured that viability at test commencement was high, as evidenced by the average 99.5 % control survival rate achieved for definitive ammonia tests (Table 3.3).

Selection of any open, but not necessarily responsive, glochidia following viability testing of a subsample resulted in an inadequate control survival rate (72.5%). Selection of glochidia that were observed to snap produced a higher average survival rate (91.4%), but was time-consuming because glochidia did not snap frequently, even under various lighting conditions (Supplementary Data, Table S3.6).

Table 3.3: Mean physico-chemical variables of test solutions across all definitive 24-hr ammonia tests (± standard deviation)

Test	Date	Species	Site	Control survival (%)	pH (Units) [a]	Temp. (°C)	EC (µS/cm) [a]	EC shift (%) [a]	Dissolved Oxygen (%) [a]
1	28/05/16	*Velesunio* sp.	MC	100	6.08 ± 0.1	27.35	99.7	2.4 ± 3.4	94.3 ± 4.0
2	31/05/16	*Velesunio* sp.	MC	100	6.11 ± 0.1	27.25	102.5	2.9 ± 3.5	92.2 ± 1.2
3	25/11/16	*Velesunio angasi*	SB	100	6.18 ± 0.1	27.5	195.5	1.2 ± 1.0	102.7 ± 0.5
4	27/05/17	*Velesunio angasi*	SB	100	6.07 ± 0.0	27.7	82.3	2.2 ± 1.3	100.8 ± 0.4
5	21/10/17	*Velesunio angasi*	SB	100	6.05 ± 0.1	27.5	131.6	3.5 ± 3.2	110.3 ± 3.9
6	06/05/17	*Velesunio angasi*	LB	96.7	6.07 ± 0.1	27.4	70.4	3.7 ± 2.0	97.4 ± 0.6
7	09/02/18	*Velesunio angasi*	LB	100	6.09 ± 0.1	27.5	76.3	1.5 ± 1.9	103.7 ± 5.5
Mean ± SD				99.5 ± 1.3	6.09 ± 0.04	27.5 ± 0.14	108.3 ± 43.6	2.5 ± 0.9	100.2 ± 6.2

[a] Values represent measurements of 3 replicate samples at 0 and 24 h from all treatments from each test (tests 1, 2, 6, 7: $n = 8$; test 3: $n = 7$; tests 4 and 5: $n = 9$). EC = electrical conductivity; MC = Magela Creek; SB = Sandy Billabong; LB = Lake Bennett.

3.3.2 Test protocol refinement

During test protocol refinement, treatments from ammonia tests that did not include any pH control showed an average 0.5 unit shift in pH over a 24-h exposure period, which was deemed unacceptable. Treatments from ammonia tests using CO_2 chambers remained within acceptable pH levels (Supplementary Data, Table S3.7). In an ammonia test using CO_2 chambers, control survival was 100%, but variable levels of survival were observed in ammonia treatments, and the response could not be clearly interpreted (Supplementary Data, Figure S3.4). As a result, the use of buffers was trialled as an alternative method of pH control. HEPES and MES buffers also maintained high control survival (≥95%) and acceptable pH control, but the MES buffer resulted in an increase in electrical conductivity (Supplementary Data, Table S3.7). On the basis of these results, the HEPES buffer was selected for definitive testing.

In earlier glochidia survival tests, 48-h survival rates were higher in Petri dishes (minimal handling 93% and extra handling 89%) compared to survival in well-plates (minimal handling 56% and extra handling 45%; Supplementary Data, Table S3.8). Survival after 24 h was lower (68%) in petri dishes containing 20 glochidia/replicate compared with Petri dishes containing 10 glochidia/replicate (77%; Supplementary Data, Figure S3.5), although this result was not significantly different (Student's *t* test, t = -0.432, 2-tailed p value = 0.667). Although both survival rates were lower than the desired >90%, they reflected early test development with results sufficient to continue further test refinement using just 10 glochidia/replicate. Although there was no statistical difference among survival rates of different ages of glochidia (< 20, 26-42 and 60-72 h; ANOVA; p = 0.923), survival was highest in the treatment containing <20-h-old glochidia with 10/replicate (77% survival). Survival was lowest (63%) in the treatment containing the 60-72-h-old glochidia (Supplementary Data, Figure S3.5).

In glochidia survival trials using Petri dishes with 10 glochidia/replicate, glochidia age of <24-h, and the improved glochidia selection method, average survival decreased from 100% at test commencement to 43% at 120-h. At 24, 48, 72, 96, and 120-h, survival was 98, 92, 84, 70, and 43% respectively. These data were comparable to previous survival data using a different test method, which also showed a decrease in survival over time (C.L. Humphey, 1986, 1987, unpublished data; Supplementary Data, Figure S3.6). A 24-h test duration was subsequently considered appropriate for *Velesunio* spp.

The comparative sensitivity of 2 different endpoints, survival and snap rate (C.L. Humphrey, 1986, 1987, unpublished data) was variable, with snap rate being more sensitive in both tests with RP2 water, and survival more sensitive in both tests with RP4 water (Supplementary Data, Figure S3.7). For consistency with international acute toxicity tests using glochidia, and because both the ecological relevance and unequivocal greater sensitivity of the snapping rate response could not be established (from earlier work), only survival was measured in the toxicity tests reported in the present study.

3.3.3 Toxicity of ammonia

The seven ammonia tests conducted using the final test protocol resulted in variable responses to TAN, both within and amongst sites. The difference in mean LC50s within each site ranged from a factor of 1.2 to a factor of 1.6 (Table 3.4). For *Velesunio* sp. from Magela Creek (tests 1 and 2), the estimated LC50 values were 7.5 and 11.5 mg/L TAN. For *V. angasi* from Sandy Billabong (tests 3, 4 and 5), the estimated LC50 values were 8.5, 13.8 and 14.2 mg/L TAN, and for *V. angasi* from Lake Bennett (tests 6 and 7), the estimated LC50 values were 6.8 and 8.2 mg/L TAN (Figures 3.2 – 3.4, and Table 3.4). When the geometric means of the LC50 values from each site (at test pH and temperature) were compared, the order of sensitivity among sites was Lake Bennett (7.4 mg/L TAN) > Magela Creek (9.3 mg/L TAN) > Sandy Billabong (11.9 mg/L TAN). The calculated NH_3 concentrations of LC50 values for each test were very low, ranging from 0.005 – 0.011 mg/L (Table 3.4). Using ANOVA, no statistically significant differences were found for LC50s among sites ($p = 0.263$), or between species ($p = 0.786$).

Using the algorithms described in Emerson *et al*. (1975) and the USEPA (2013), the geometric means were converted to toxicity estimates of 9.2, 11.3 and 14.8 mg/L TAN at pH 7 and temperature 20°C, and 2.1, 2.6 and 3.5 mg/L TAN at pH 8 and temperature 20°C, to compare with other published data (Supplementary Data, Table S3.9). Two of these converted ammonia toxicity estimates (Lake Bennett and Magela Creek) were lower (more sensitive) than previously reported toxicity estimates for temperate freshwater mussels (Figure 3.5 and Supplementary Data, Table S3.9). All 3 toxicity estimates were among the most sensitive when compared with converted ammonia toxicity estimates from other tropical freshwater taxa (Figure 3.6). The % coefficient of variation of adjusted LC50 values at pH 7 and temperature 20°C was calculated for each site as a further measure of variability, and remained within 30% for each site, ranging from 13.8 to 29.3% (Table 3.4).

Table 3.4: Summary of the ammonia toxicity estimates in total ammonia nitrogen and NH_3 for tests with *Velesunio* sp. (Magela Creek), and *Velesunio angasi* (Sandy Billabong and Lake Bennett)

Species	Site	Test	Total Ammonia Nitrogen (mg/L)		NH_3 (mg/L) [c]		%CV [d]	Mean toxicity value ratio [e]
			LC50 (95% CI) [a]	Geometric mean	LC50 (at pH 7, 20°C) [b]	LC50 (at test conditions)		
Velesunio sp.	Magela Creek	1	7.5 (6.8 – 8.2)	9.3	9.2	0.006	29.3	1.5
		2	11.5 (8.3 – 15.6)		14.0	0.010		
V. angasi	Sandy Billabong	3	8.5 (7.9 – 9.3)		10.7	0.009		
		4	13.8 (12.0 – 15.9)	11.9	17.4	0.011	25.5	1.6
		5	14.2 (12.2 - 16.4)		17.4	0.011		
V. angasi	Lake Bennett	6	6.8 (5.9 - 7.8)	7.4	8.3	0.005	13.8	1.2
		7	8.2 (6.8 - 9.6)		10.1	0.007		

[a] Values are shown for test conditions, pH ~6.0, and water temperature ~27.5°C

[b] Values are shown for test pH and temperature adjusted to pH and 20°C using the USEPA (2013) algorithms. The % coefficient of variation and mean toxicity value ratio of the median lethal concentrations (LC50s) for each site are shown as a measure of variability between species and sites. Analysis of variance showed no statistically significant difference among sites ($p = 0.263$) or between species ($p = 0.786$).

[c] Calculated using speciation equations of Emerson *et al.* (1975).

[d] %CV = standard deviation/mean of LC50 values adjusted to pH 7, 20°C using algorithms of USEPA (2013).

[e] Mean toxicity value ratio = maximum LC50/minimum LC50 values (at pH 7, 20°C).

CI = confidence interval.

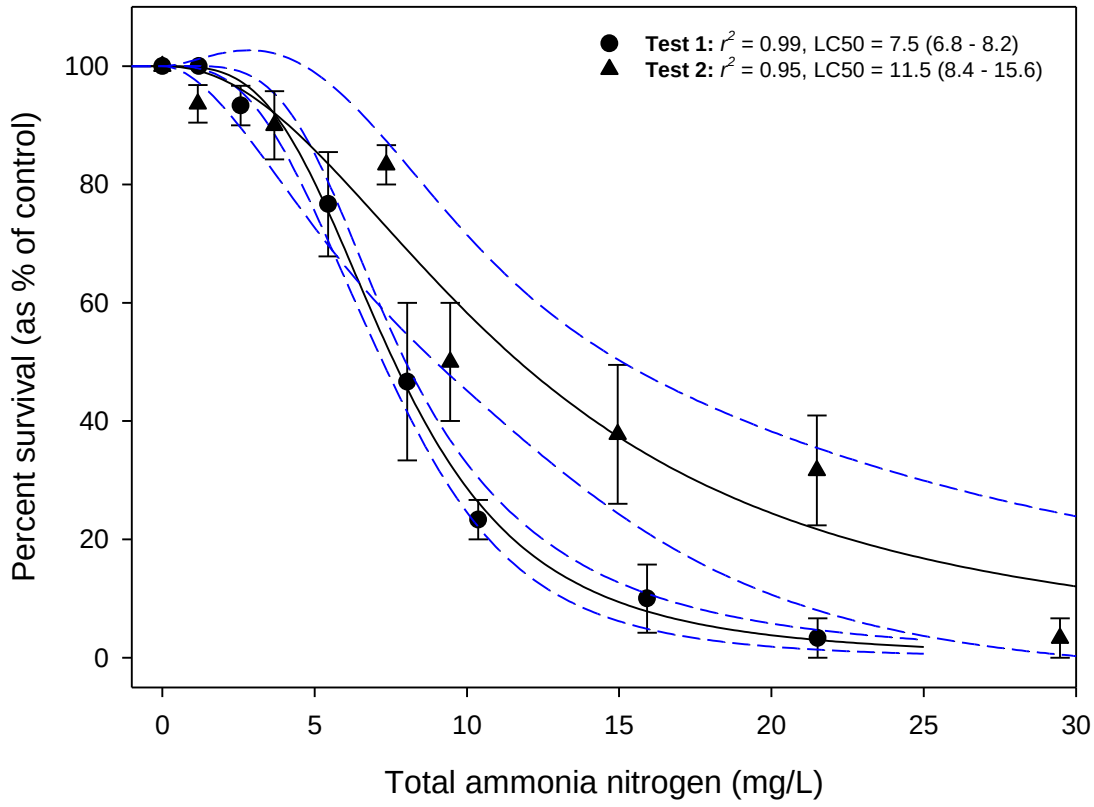

Figure 3.2: Percentage survival of *Velesunio* sp. glochidia from Magela Creek following 24-h exposures to ammonia using the final test protocol. Data points represent the mean ± standard error of three replicates from each of the 2 tests. The 95% confidence intervals for each test are represented by broken lines. Toxicity estimates were determined using 3-parameter logistic models. LC50 = median lethal concentration.

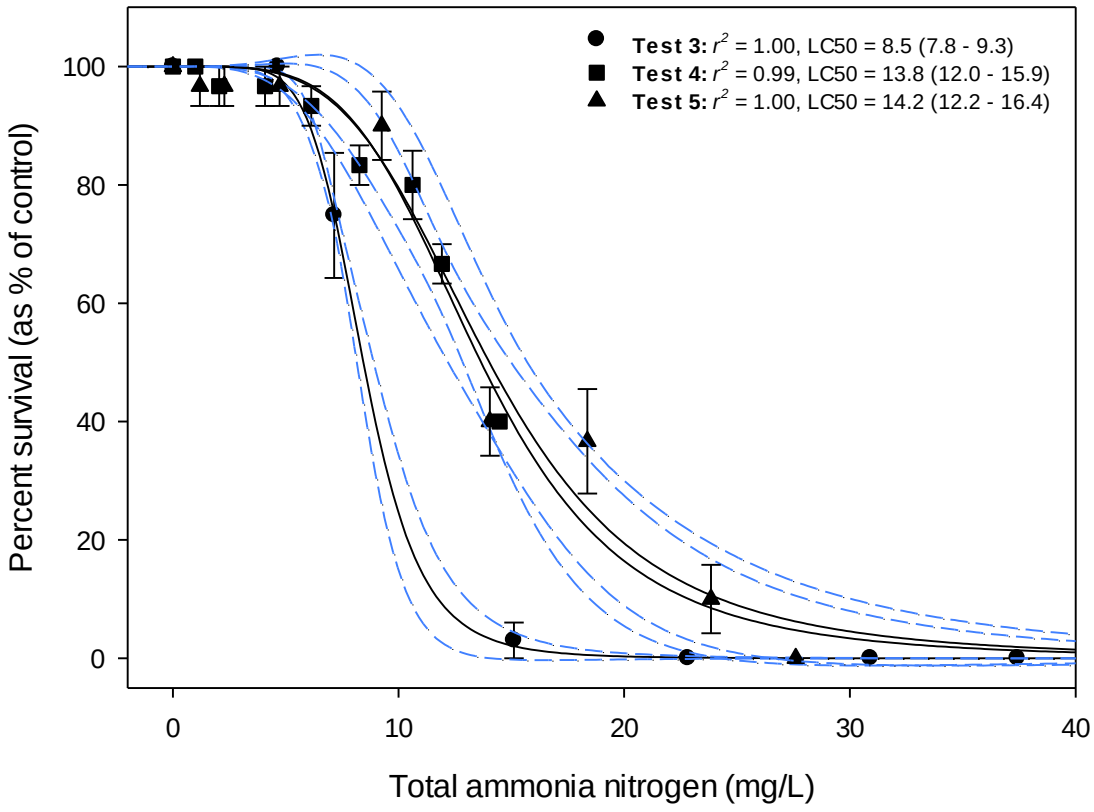

Figure 3.3: Percentage survival of *Velesunio angasi* glochidia from Sandy Billabong following 24-h exposures to ammonia using the final test protocol. Data points represent the mean ± standard error of three replicates from each of the 3 tests. The 95% confidence intervals for each test are represented by broken lines. Toxicity estimates were determined using 3-parameter logistic models. LC50 = median lethal concentration.

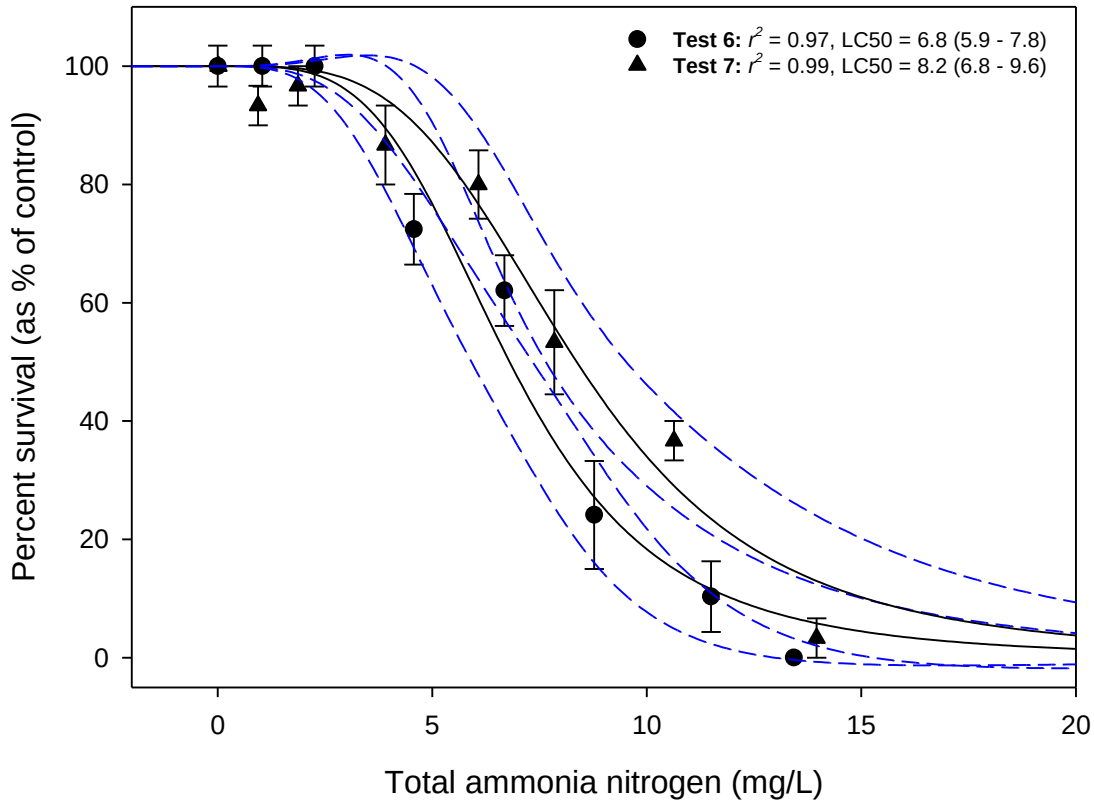

Figure 3.4: Percentage survival of *Velesunio angasi* glochidia from Lake Bennett following 24-h exposures to ammonia using the final test protocol. Data points represent the mean ± standard error of three replicates from each of the 2 tests. The 95% confidence intervals for each test are represented by broken lines. Toxicity estimates were determined using 3-parameter logistic models. LC50 = median lethal concentration.

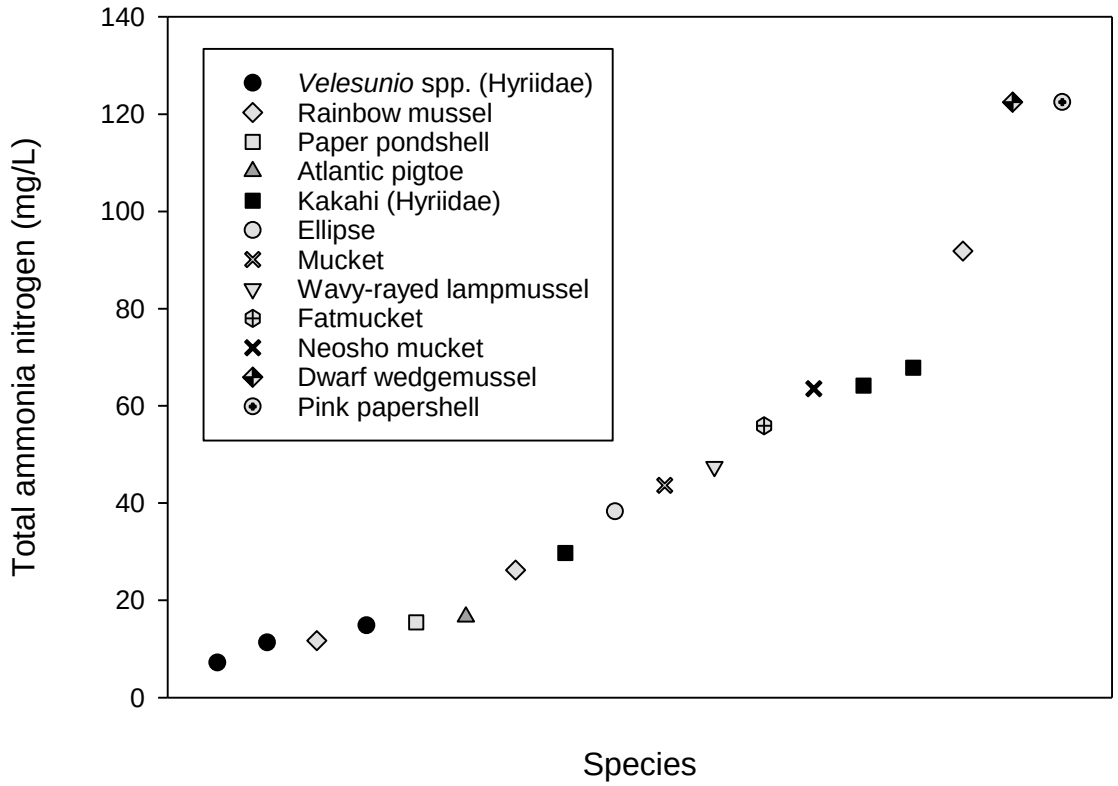

Figure 3.5: Comparison of the sensitivity of *Velesunio* sp. 24-h ammonia median lethal concentration (LC50) data from the present study (black circles) with available 24-h ammonia LC50 data for temperate freshwater mussel species. All data were adjusted to pH 7 and 20°C.

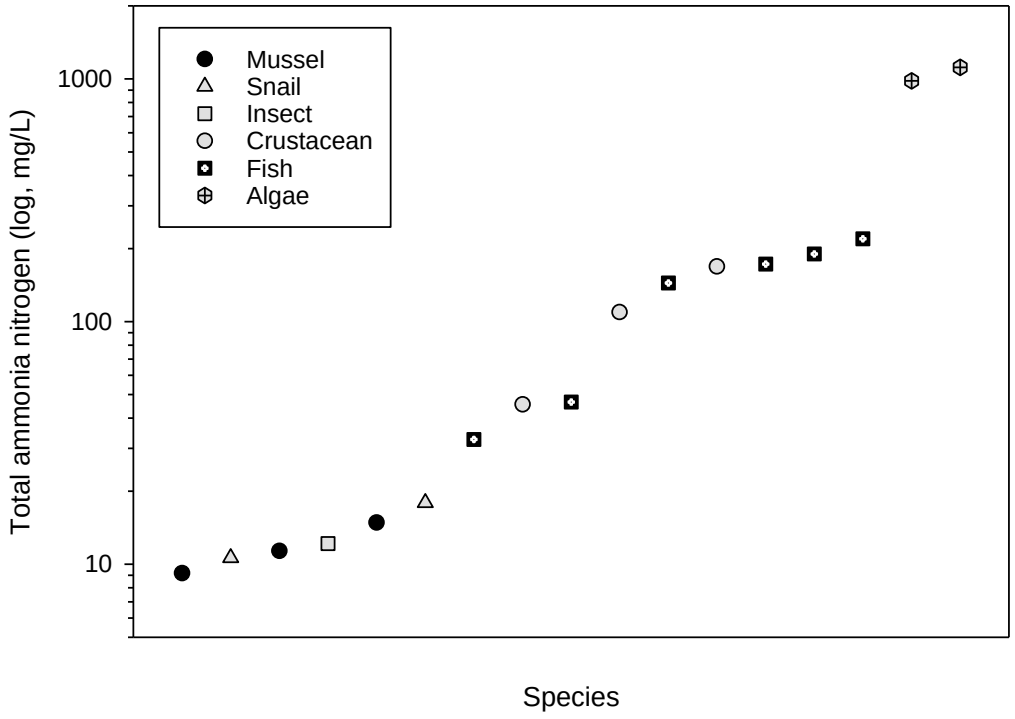

Figure 3.6: Comparison of the sensitivity of *Velesunio* sp. ammonia median lethal concentration (LC50) data from the present study (black circles) with available acute ammonia EC50/LC50 data for other tropical freshwater taxa (all data adjusted to pH 7 and 20°C). Adapted from Wang and Leung (2015); Økelsrud and Pearson (2007); Økelsrud (2004).

3.4 Discussion

3.4.1 Test protocol refinement

The toxicity test protocol refined in the present study had many benefits that make it suitable as a standard protocol for *Velesunio* spp., including its short duration, ease of test organism handling, and ease of endpoint measurement. The difficulties associated with dependency on wild-collected mussels for glochidia (e.g., site access, seasonal breeding cycles, and availability of gravid females) prevent the toxicity test from being routinely applied year-round. Further research into cultivating *Velesunio* spp. in the laboratory could potentially provide a more accessible source of glochidia for toxicity testing; this was beyond the scope of this study. Cultivation techniques for propagating juvenile mussels, which also provide a source of glochidia for toxicity testing, have been developed successfully in larger US facilities for various temperate mussel species (ASTM 2006). Exposing mussels in shallow containers of water to expel glochidia was successful in producing viable glochidia

for testing, but the expulsion was often protracted, not always yielding large numbers at the outset (Supplementary Data, Table S3.1). Alternative methods include the extraction of glochidia by prising open the mussel shells and flushing the gills with a syringe (Wang *et al*. 2007a; Fritts *et al*. 2014; Markich 2017). Advantages of flushing out the glochidia might include production of larger numbers of mature glochidia for immediate use in a test. The age of these glochidia would therefore be more precisely pinpointed at the start of the test. However, this method may result in harm to the adult mussel by damaging the gills or marsupia. McLeod *et al*. (2017) had similar concerns when testing the imperilled species, *Ptychobranchus*, and instead induced release by raising the temperature of the tank slowly. Although ASTM (2006) recommends flushing the marsupium in this manner, it was extremely difficult to prise open the valves of *Velesunio* sp. to access the inner gills without causing damage to the adductor muscles. The tight shell seal of this species may be an anatomical feature for its adaptation to dormancy in the moist sand banks of seasonally-flowing creeks in the Alligator Rivers Region during the annual dry season (typically May–Nov). One of us (C.L. Humphrey) notes that for *Velesunio* sp., a tight hermetic seal is created with the mussel valves to prevent water loss during this dormancy period, involving a mucous seal and strong adductor muscles to keep the valves tightly shut.

The glochidia viability and selection procedures used throughout the present study resulted in the production of healthy glochidia for ammonia toxicity testing, with very high control performance (average 99.5% survival after 24 ; Table 3.3). The reliability of the final selection method based on the physical appearance of glochidia was confirmed through consistent observations of high survival of control glochidia after 24 h. This method did not expose the glochidia to long periods of bright lighting, handling, or excessive changes in water temperature during selection under the microscope.

Many studies cited by ASTM (2006) have used viability testing to evaluate subsamples of glochidia obtained from each mussel. If ≥90% viability is achieved, the remaining glochidia are pooled together to be used in toxicity tests. In this way, control viability at the start of the test is known, and the survival of each treatment can be adjusted as a percentage of control survival (Wang *et al*. 2007a). Viability testing using a salt solution was useful in this study to check the condition of glochidia from each adult female prior to selection for tests. However, small numbers of glochidia (10) were used in each treatment replicate in toxicity tests, because large numbers were not always available for testing. This sample size constraint made refinement of glochidia selection necessary to ensure maximum viability for testing.

The control of pH during static-renewal toxicity testing was very important because pH changes can alter the toxicity of test solutions. In particular, the toxicity of ammonia can increase 10-fold per unit rise in pH (Mount and Mount 1991; USEPA 2013). Ideally, test pH should be maintained at levels that are representative of the receiving water when the purpose of a toxicity test is to predict potential impacts of an effluent (Mount and Mount 1991). Likewise, dilution water characteristics should replicate the site of interest in site-specific evaluations (ASTM 2006). The Magela Creek water used throughout the present study is typically poorly buffered and prone to pH shifts. The use of CO_2 for pH control can be advantageous because it mimics the natural carbonate buffering system maintaining the natural conditions at that pH. This buffering system results in an ongoing pH control throughout the test (Mount and Mount 1991). The use of CO_2 as a buffer was successful in controlling pH but ultimately resulted in variable survival among ammonia treatments, and the data could not be used for generation of a concentration-response curve to estimate an LC50. Control survival of 100% was achieved using chambers charged with 0.26 to 0.34 % CO_2 gas after the glochidia selection method was refined, suggesting that the CO_2 itself did not cause toxicity to glochidia. Although elevated concentrations of CO_2 can potentially harm aquatic organisms, it has been reported that up to 10% CO_2 should not cause any adverse effects, and that using the correct mixture should maintain the CO_2 concentration in the initial sample (Elphick *et al*. 2005). It was difficult to determine the cause of the variable survival, and further investigation may be warranted. Using CO_2 as a buffer also posed challenges in determining the amount of CO_2 required, due to the fluctuating pH (~5.5–6.5), of natural Magela Creek water throughout the year, especially when Magela Creek water was close to pH 6. As a result of inconsistencies when CO_2 was used as a buffer, alternative buffers were investigated. The MES and HEPES buffers produced similar survival rates but MES raised the electrical conductivity, and so HEPES was chosen as the preferred buffer. The HEPES buffer did not affect glochidia survival, and maintained the pH within the desired range.

In the literature, the number of glochidia used per treatment replicate varies greatly, ranging from 10 – 3000/replicate, with the larger replicate numbers assessed by taking subsamples (ASTM 2006). Because of constraints in acquiring adequate numbers of wild-collected mussels with mature larvae, it was necessary to use the lowest number of glochidia possible whilst still maintaining statistical rigour.

Survival of *Velesunio* spp. glochidia in the water column after expulsion from adult females is relatively short. In the present study, 98% survival of controls was achieved after 24 h, declining to 92% after 48 h, and dropping below 90% thereafter (Supplementary Data,

Figure S3.6). The survival data were comparable to previous unpublished data for *Velesunio* sp. (C.L. Humphrey, 1986, 1987; Supplementary Data, Figure S3.6), where higher survival rates were achieved due to test design (using a flow-through system and no handling of glochidia). Accordingly, glochidia that were as young as possible (<24-h post expulsion) were used for testing, representing an optimal age for toxicity testing.

The <24-h age for testing is congruent with ASTM (2006) test acceptability requirements, which recommend the use of glochidia <24-h old and report glochidial ages ranging from <2 to <48-h old. Although some studies support a 48-h test duration with glochidia (Wang *et al*. 2007a), it is recommended that acute test durations should be 24 h for most species, but can be up to 48 h if >90% control survival is achieved (ASTM 2006). Test duration was dependent on the survival time of glochidia in the water, with longer survival times observed in species from cooler climates (ASTM 2006). Although high survival rates were still achieved after 48 h for *Velesunio* spp. (Supplementary Data, Figure S3.6), the decrease in survival after 24 h demonstrated that a shorter test duration was appropriate for this tropical species.

The snapping rate endpoint measured by Humphrey (1987a) was based on the observation that a snapping response can be produced by exposure to an intense light source and that a decline in snapping rates when exposed to a toxicant can be measured and interpreted as a loss of viability. This endpoint was investigated in the present study using test apparatus different from that employed by Humphrey (1987a), but we decided not to use it. A consistent glochidial snapping rate could not be established under various lighting conditions, and individual measurement was too time-consuming. Furthermore, comparative sensitivity of the survival and snap rate endpoints from unpublished data gathered in 1986 and 1987 (as shown earlier and now analysed in the present study; Supplementary Data, Figure S3.7) showed that the snapping rate response was measureable as a behavioural endpoint but was not consistently more sensitive than the survival endpoint. Thus, reduced snap rate as a precursor to mortality cannot be assumed for all test waters and may require further investigation.

The survival endpoint as measured using salt (NaCl) stimuli to assess the valve closure response was considered ecologically relevant in the present study because it indicated the ability of glochidia to attach to a host fish and metamorphose into the juvenile life stage (Fritts *et al*. 2014). Glochidia are considered functionally dead if their valves fail to close after a suitable stimulus (in this case, exposure to the salt solution), because they would be unable to attach to a host fish (ASTM 2006).

3.4.2 Ammonia toxicity

Intra- and interpopulation variation in toxicity responses to ammonia was evident for *Velesunio* spp, with varying sensitivity observed within and between populations (Table 3.4 and Figures 3.2–3.4). Comparative information on intra- and interlaboratory variability in toxicity using mussel glochidia is limited. Wang *et al*. (2007b) conducted multiple toxicity tests with glochidia and juvenile mussels within a single laboratory and among 5 laboratories, using several toxicants including ammonia. Variability was expressed as the mean % coefficient of variation, and for ammonia tests with glochidia of 2 species, the intralaboratory % coefficient of variation of 4 tests with mucket (*Actinonaias ligamentina*) was 25% at 24 h, and 27% for 6 tests with fatmucket (*Lampsilis siliquoidea*) also at 24 h. This variability was considered low (Wang *et al*. 2007b) and is comparable to that reported in the present study (% coefficient of variation values of 13.8–29.3; Table 3.4). Raimondo *et al*. (2016) developed a toxicity database of mussel species to examine intra- and interlaboratory variability for acute toxicity tests, and used mean toxicity value ratios as a measure of variability. Intralaboratory variability for glochidia with ammonia averaged a factor of 1.5, which was also comparable to that reported in the present study (average factors of 1.2–1.6; Table 3.4). The variation in sensitivity may have been influenced by several factors, such as differences in the age and maturity of glochidia used in each test (between 1 and 24 h after expulsion from adult mussels), genetic variability between glochidia batches, different seasons for testing, and differences in physicochemical properties among the batches of diluent water used. A major contributing factor to the high sensitivity of Lake Bennett mussels may be the differences in physicochemistry between the Magela Creek water diluent water used and higher ionic strength waters of Lake Bennett (higher in average pH [7.1 ± 0.3], conductivity [41.0 ± 8.0 µS/cm] and hardness [14.7 ± 2.1 mg/L as CaCO3], and differing from Magela Creek water in several major ions; Supplementary Data, Table S3.5). Test exposures typically commenced on the same day that glochidia were isolated from the adult mussel, which limits the time available to acclimate glochidia to the dilution water (Ingersoll *et al.* 2007). Rapid changes in water conditions may cause osmotic stress, which in turn could increase the sensitivity of glochidia to contaminants.

The test temperature (~27.5 °C) and pH (~6.0) used in the present study were based on typical ambient water quality conditions in Magela Creek (Klessa 2000) and were similar to site-specific test conditions used for other species of the Alligator Rivers Region routinely used in toxicity testing (Riethmuller *et al.* 2003). Magela Creek water is typically slightly to

moderately acidic (pH ~5.5–6.5), and very low in electrical conductivity (~5–20 µS/cm), water hardness (~3–6 mg/L as $CaCO_3$) and alkalinity (5–10 mg/L as $CaCO_3$; van Dam *et al*. 2010). The relationship between ammonia toxicity and both pH and temperature has been calculated in algorithms provided by Emerson *et al*. (1975). Generally, NH_3 increases 10-fold/pH unit increase, and 2-fold for each 10 °C temperature rise between 0 and 30 °C (USEPA 2013). According to the chemistry underpinning ammonia speciation (Emerson *et al*. 1975), the less toxic ammonium ion (NH_4^+) would have been dominant (>99%) under our test conditions. Increasing the pH of the test solutions would increase the proportion of un-ionised ammonia (NH_3) and has been shown to result in higher toxicity to juvenile mussels (Wang *et al*. 2008). The fact that the toxicity of ammonia to *Velesunio* sp. in the present study was high in comparison with toxicity reported for other species tested at a higher pH (and lower temperature), suggests that the ammonium ion can exert toxic effects at pH 6.0 (ANZECC/ARMCANZ 2000). This is supported by the very low NH_3 concentrations calculated for LC50 values for each test in the present study (Table 3.4) in comparison with reported NH_3 concentrations from previous studies (Supplementary Data, Table S3.9). The very soft Magela Creek water may also have contributed to the increased toxicity of ammonia during the present study. The toxicity of ammonia to the amphipod *Hyalella azteca* has been shown to increase with decreasing water hardness, suggesting that *H. azteca* was sensitive to NH_4^+ in soft water (Ankley *et al.* 1995). The pH-ammonia toxicity relationship was recently assessed for the tropical green hydra, *Hydra viridissima*, using Magela Creek water adjusted to a pH range between 6.0 and 8.5. Although increasing pH increased the sensitivity of *H. viridissima* to ammonia, no conclusions could be drawn on the effects of ionic concentrations on ammonia toxicity (Mooney *et al.* 2018). Further toxicity testing with ammonia and under various water conditions would be needed to determine whether ammonia toxicity to *Velesunio* spp. is influenced by the interactions among pH, temperature, and water hardness.

Although no comparative ammonia data are available for tropical freshwater mussel species, there are data for temperate mussel species and other tropical taxa to provide a point of comparison for the present study. Based on adjusted LC50 values, *Velesunio* spp. were more sensitive than all but one temperate mussel species in the temperate freshwater mussel dataset (Figure 3.5 and Supplementary Data, Table S3.9). When compared with the tropical freshwater dataset for ammonia, *Velesunio* spp. were among the most sensitive to ammonia in comparison with reported estimates for 13 tropical taxa, which ranged from 10.6 mg/L for a snail species (*Sulcospira hainanesis*) to 1117 mg/L for an algal species (*Chlorella vulgaris*) after conversion to pH 7 and 20 °C (Figure 3.6). This finding further confirmed the sensitivity

of *Velesunio* spp. and indicated its high sensitivity to ammonia in comparison with other tropical species.

For estimating a chronic value for ammonia from acute data, the USEPA (2013) provide a Unionidae acute-to-chronic ratio (ACR) of 15.52, which is applied to the median acute value to predict chronic sensitivity when measured chronic toxicity data are limited. Applying this ACR to the acute LC50 for the present study (adjusted LC50 geometric mean of 2.78 mg/L TAN at pH 8 and 20 °C) results in an estimated chronic value of 0.18 mg/L, which is lower than the 99% species protection level trigger value of 0.32 mg/L (at pH 8 and 20 °C) for ecosystems with high conservation value provided by the ANZECC/ARMCANZ (2000), indicating that the trigger value would not be adequately protective using this ACR. This further highlights the need for toxicity testing of juvenile *Velesunio* spp. using chronic, sublethal exposures, which would provide an actual chronic value that could be used in determining a more relevant site-specific guideline value for ammonia. Future work will involve the development of a chronic toxicity test for freshwater mussels that can be used to assess a range of contaminants.

3.5 Conclusion

The present study resulted in the successful refinement of an acute toxicity test to determine the sensitivity of northern Australian freshwater mussels to water-borne toxicants. The first acute toxicity estimates for ammonia for a tropical freshwater mussel species were derived using the refined test protocol, and this protocol can now be applied to assess other toxicants. The acute toxicity estimates indicated that *Velesunio* spp. were among the most sensitive species to ammonia compared with temperate freshwater mussel species, and were among the most sensitive species compared with tropical freshwater species from other taxa. Test repeatability was consistent with that reported for other comparable mussel studies, providing additional confidence in the rigor of the protocol. Future work will include the development of a chronic toxicity test protocol using the growth rate of juvenile mussels as an endpoint, which is likely to provide a more relevant toxicity estimate for ammonia with this species. These toxicity estimates will contribute to the development of a tropical guideline value for ammonia, and may be incorporated in revisions of national and/or international water quality guideline values.

Acknowledgment

The authors thank the ecotoxicology team and Jabiru field station staff at the Supervising Scientist Branch, Australian Department of the Environment and Energy, for their support and assistance with field collections for this study. Collections on public land were conducted under special permit 2015-2016/S17/3380 issued by the Department of Primary Industry and Fisheries, Northern Territory, Australia and permit 57834 issued by the Parks and Wildlife Commission, Northern Territory, Australia. Field collections within the Alligator Rivers Region were conducted with permission under project RES-2015-025, PAN-eriss Protocols 2015-16. Field collections at Lake Bennett were conducted with permission from the caretaker of Lake Bennett. During the course of the present study, L.S. Kleinhenz was the recipient of an Australian Government Research Training Program scholarship administered through Royal Melbourne Institute of Technology University.

Supplementary Data

FIGURES

Figure S3.1: In definitive tests, glochidia were selected individually under the microscope by observation of physical appearance and behaviour of glochidia with opened valves. The picture illustrates the visible adductor muscles, well developed teeth, and translucent appearance used as indicators of health and viability of mature glochidia. Intermittent valve closure (snapping) was also used as an indicator of viability.

Figure S3.2: The set-up for the test development volume and chamber trial using a 12-well plate submerged in a 700 mL plastic container.

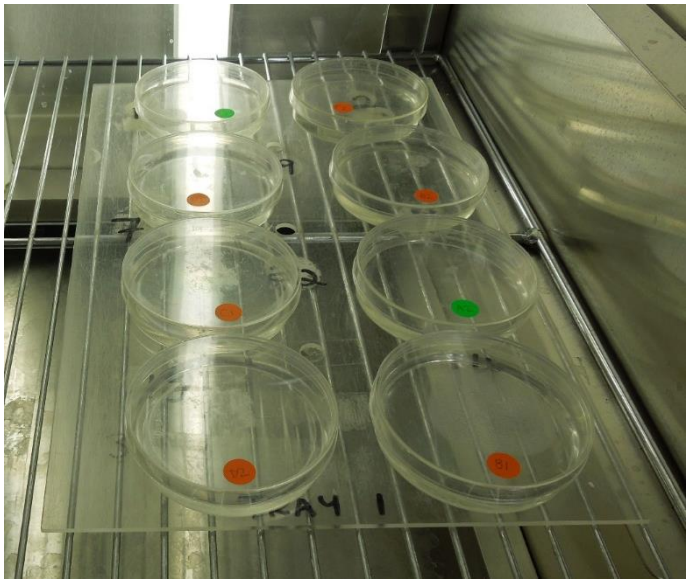

Figure S3.3: The set-up for the test development volume and chamber trial using 30 mL of test solution in 9 cm petri dishes.

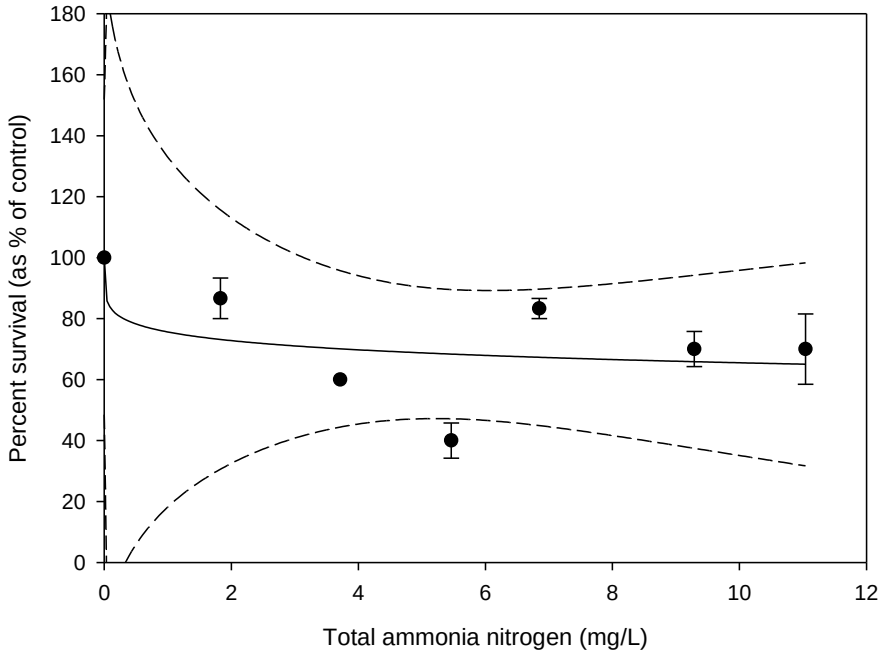

Figure S3.4: Percent survival of glochidia following 24-h exposure to ammonia (with pH control: CO_2 gas, 2600-3400 ppm).

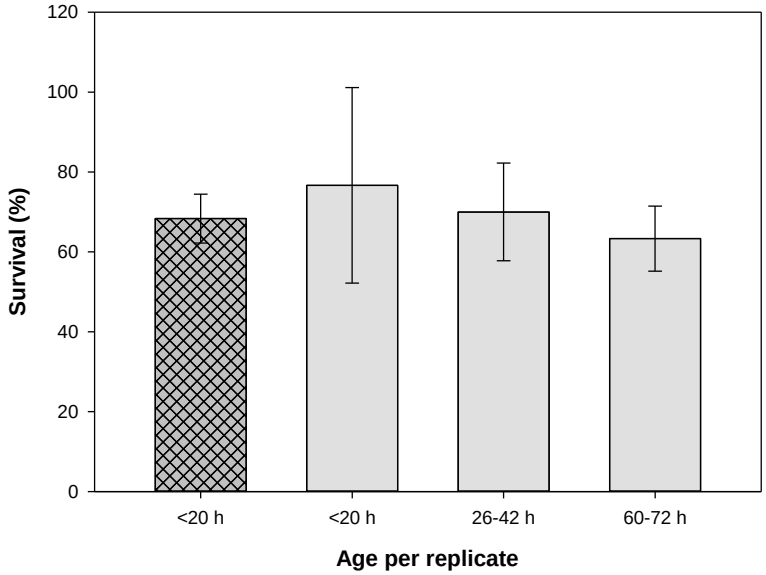

Figure S3.5: The 24-h survival of four different ages of glochidia after release. Hatched column represents 20 glochidia per replicate. Unhatched columns represent 10 glochidia per replicate. Data represents the mean ± se of three replicates.

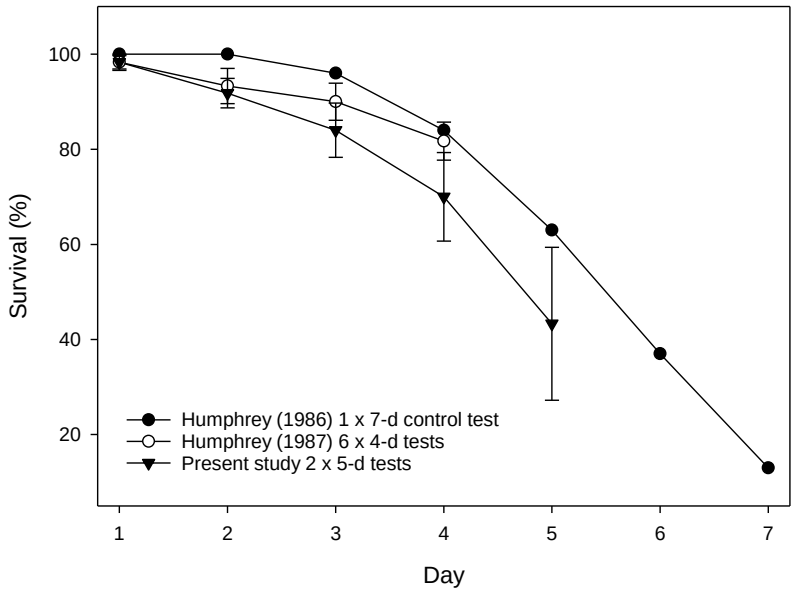

Figure S3.6: Glochidia survival comparison over 7 days (Humphrey 1986), 4 days (Humphrey 1987), and 5 days (present study) showing decrease in survival over time in the water column. Glochidia age at test commencement <24-h. Data from Humphrey (1987) represents the mean ± se of five tests with one replicate using *V. angasi* from Mudginberri Billabong, and data from the present study represents the mean ± se of two tests with three replicates, using *V. angasi* from Sandy Billabong.

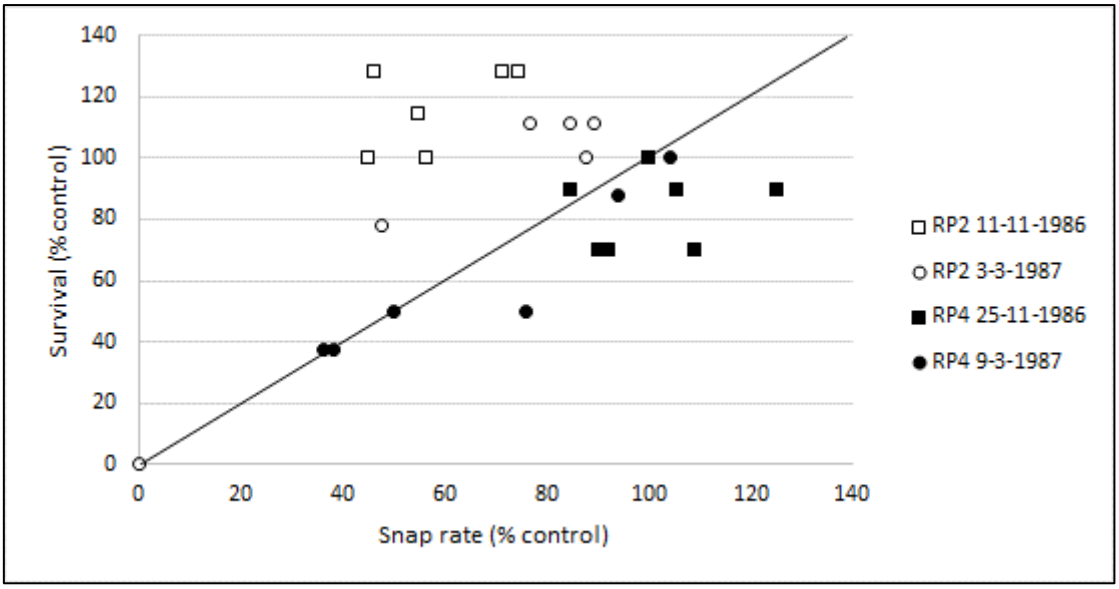

Figure S3.7: Data from four mine retention pond-water tests (unpublished data from Humphrey 1986 and 1987) comparing the sensitivity of snap rate and survival endpoints. Data points lying above the 1:1 line (RP2 waters) indicate higher sensitivity. Snap rate was the more sensitive endpoint for RP2 waters, while survival was the more sensitive endpoint for RP4 waters.

TABLES

Table S3.1: Observations of larval maturity in mussels collected from various field sites throughout this study.

Exposure date	**Source of mussels** [a]	**# of mussels collected**	**# with eggs or embryos only**	**# with immature glochidia** [b]	**# with mature glochidia** [c]	**Mature glochidia (%)**
22/10/15	MB	6	-	-	1	16.7
23/10/15	SB	40	-	-	2	5.0
27/01/16	MD	18	-	9	2	11.1
23/02/16	MD	41	11	4	-	0.0
14/04/16	MC	80	-	6	8	10.0
13/05/16	MC	50	3	13	6	12.0
27/05/16	MC	46	-	3	2	4.3
28/06/16	MC	13	-	-	-	0.0
31/10/16	MD	41	-	2	1	2.4
16/11/16	BB	47	-	-	-	0.0
24/11/16	SB	42	5	10	3	7.1
01/12/16	MB	78	2	5	-	0.0
10/01/17	LB	45	1	10	7	15.6
09/02/17	LB	40	4	6	2	5.0
03/03/17	GC	29	-	5	2	6.9
15/03/17	LB	50	1	17	6	12.0
18/04/17	GC	44	-	-	4	9.1
05/05/17	LB	86	-	26	7	8.1
26/05/17	SB	51	5	9	8	15.7
15/06/17	SB	64	-	6	6	9.4
20/10/17	SB	55	-	18	5	9.1
24/10/17	MB	75	-	21	5	6.7
Mean (SD)		47.3 (20.6)	2.5 (3.2)	7.7 (7.3)	3.7 (2.7)	**7.6** (5.2)

[a] MB = Mudginberri Billabong, SB = Sandy Billabong, MD = Manton Dam, MC = Magela Creek, BB = Bowerbird Billabong, LB = Lake Bennett, GC = Gulungul Creek.

[b] Mature glochidia were identified as those free of a membrane, open at the hinge, with visible teeth, and displaying intermittent closing of the valves.

[c] Immature glochidia were identified as those closed at the hinge and still encased in a membrane

Table S3.2: Nominal and measured ammonia concentrations for all definitive ammonia tests using the final test protocol.

Test / treatment (internal code)	Nominal (mg/L TAN)	Measured concentration at start of test (mg/L TAN)	Measured concentration at end of test (mg/L TAN)	Average measured (mg/L TAN)	% change to nominal	% change start / end
1. 1524M						
A	0	0.03	0.04	0.04		
B	1.25	1.20	1.19	1.20	-4.40	-0.63
C	2.5	2.69	2.45	2.57	2.70	-8.75
D	5	5.53	5.35	5.44	8.80	-3.25
E	7.5	8.01	8.06	8.03	7.10	0.56
F	10	11.10	9.64	10.37	3.70	-13.15
G	15	16.08	15.75	15.92	6.10	-2.05
H	20	20.56	22.48	21.52	7.60	9.34
2. 1525M						
A	0	0.03	0.02	0.02		
B	1	1.23	1.11	1.17	16.7	-9.93
C	3	3.72	3.64	3.68	22.60	-2.26
D	6	7.54	7.16	7.35	22.49	-4.95
E	8	9.31	9.66	9.49	18.60	3.78
F	12	15.07	14.83	14.95	24.60	-1.59
G	18	22.75	20.23	21.49	19.40	-11.08
H	24	28.80	30.14	29.47	22.80	4.67
3. 1541M						
A	0	0.02	0.05	0.03		
B	4	4.68	4.59	4.64	15.88	-1.92
C	6	6.96	7.33	7.15	19.08	5.32
D	12	14.97	15.24	15.11	25.88	1.80
E	18	22.72	22.93	22.83	26.81	0.92
F	24	31.06	30.76	30.91	28.79	-0.97
G	36	35.93	38.90	37.42	3.93	8.27
4. 1595M						
A	0	0.01	0.03	0.02		
B	1	0.94	1.04	0.99	-1.20	10.21
C	2	1.98	2.12	2.05	2.40	7.29
D	4	3.87	4.28	4.08	1.90	10.54
E	6	5.87	6.38	6.13	2.10	8.79
F	8	8.02	8.50	8.26	3.20	5.99
G	10	9.98	11.26	10.62	6.20	12.83
H	12	11.33	12.50	11.92	-0.70	10.38
I	14	13.92	15.04	14.48	3.40	8.05

Table S3.2: (Continued)…

Test / treatment (internal code)	Nominal (mg/L TAN)	Measured concentration at start of test (mg/L TAN)	Measured concentration at end of test (mg/L TAN)	Average measured (mg/L TAN)	% change to nominal	% change start / end
5. 1638M						
A	0	0.00	0.01	0.01		
B	1.25	1.24	1.17	1.20	-3.80	-5.26
C	2.5	2.28	2.29	2.28	-8.80	0.44
D	5	4.73	4.73	4.73	-5.40	0.00
E	10	9.38	9.12	9.25	-7.50	-2.77
F	15	14.10	13.98	14.04	-6.40	-0.85
G	20	18.52	18.20	18.36	-8.20	-1.73
H	25	23.90	23.80	23.85	-4.60	-0.42
I	30	27.06	28.14	27.60	-8.00	3.99
6. 1586M						
A	0					
B	1	1.00	1.07	1.04	3.60	6.79
C	2	2.20	2.32	2.26	13.00	5.08
D	4	4.48	4.66	4.57	14.20	3.93
E	6	6.40	6.96	6.68	11.30	8.82
F	8	8.69	8.85	8.77	9.60	1.84
G	10	11.48	11.52	11.50	15.00	0.35
H	12	13.13	13.73	13.43	11.90	4.57
7. 1679M						
A	0	0.03	0.01	0.02		
B	1	0.97	0.91	0.94	-6.20	-5.80
C	2	1.82	1.92	1.87	-6.50	5.49
D	4	4.06	3.76	3.91	-2.30	-7.30
E	6	6.28	5.88	6.08	1.30	-6.31
F	8	7.90	7.78	7.84	-2.00	-1.62
G	11	10.45	10.82	10.64	-3.30	3.58
H	14	14.03	13.89	13.96	-0.30	-1.00
				Total Mean	6.46	1.20

Table S3.3: Water chemistry measurements of blanks and control treatments for each test with Magela Creek glochidia. NM = not measured.

Test			1. Magela Creek (1524M)			2. Magela Creek (1525M)		
	Units	Detection limit	Blank	Pro-Blank	Control	Blank	Pro-Blank	Control
Date sampled			28/5/16	-	28/5/16	31/5/16	-	31/5/16
Date analysed			14/7/16	-	14/7/16	14/7/16	-	14/7/16
Aluminium	µg/L	0.1	<0.1	NM	15	0.1	NM	11
Cadmium	µg/L	0.02	<0.02	NM	<0.02	<0.02	NM	<0.02
Cobalt	µg/L	0.01	<0.01	NM	0.05	<0.01	NM	0.06
Chromium	µg/L	0.1	<0.1	NM	<0.1	<0.1	NM	<0.1
Copper	µg/L	0.01	0.02	NM	0.07	0.06	NM	0.07
Iron	µg/L	1	<1	NM	73	<1	NM	54
Manganese	µg/L	0.01	0.05	NM	2	<0.01	NM	2
Nickel	µg/L	0.01	<0.01	NM	0.2	2	NM	0.1
Lead	µg/L	0.01	<0.01	NM	0.01	<0.01	NM	0.04
Selenium	µg/L	0.2	<0.2	NM	<0.2	<0.2	NM	<0.2
Uranium	µg/L	0.001	0.001	NM	0.007	0.001	NM	0.02
Zinc	µg/L	0.1	<0.1	NM	0.4	<0.1	NM	0.5
Calcium	mg/L	0.1	<0.1	NM	0.2	<0.1	NM	0.2
Magnesium	mg/L	0.1	<0.1	NM	0.9	<0.1	NM	0.9
Sodium	mg/L	0.1	<0.1	NM	1.2	<0.1	NM	1.2
Sulphate	mg/L	0.5	<0.5	NM	<0.5	<0.5	NM	<0.5

Table S3.4: Water chemistry measurements of blanks, pro-blanks and control treatments for each test with Sandy Billabong glochidia.

Test			3. Sandy Billabong (1541M)			4. Sandy Billabong (1595M)			5. Sandy Billabong (1638M)		
	Units	Detection limit	Blank	Pro-Blank	Control	Blank	Pro-Blank	Control	Blank	Pro-Blank	Control
Date sampled			25/11/16			27/5/17			21/10/17		
Date analysed			8/12/16			29/6/17			16/11/17		
Aluminium	µg/L	0.1	<0.1	<0.1	10	<0.1	0.2	14	8.1	2.1	8.6
Cadmium	µg/L	0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Cobalt	µg/L	0.01	<0.01	<0.01	0.07	<0.01	<0.01	0.05	<0.01	0.16	0.06
Chromium	µg/L	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	<0.1
Copper	µg/L	0.01	<0.01	<0.01	0.1	0.04	0.06	0.13	0.44	0.16	0.18
Iron	µg/L	1	<1	<1	72	<1	<1	50	5	11	83
Manganese	µg/L	0.01	<0.01	<0.01	3.0	<0.01	<0.01	1.3	10	360	4
Nickel	µg/L	0.01	<0.01	<0.01	0.1	0.07	0.04	0.11	0.31	0.16	0.18
Lead	µg/L	0.01	<0.01	<0.01	0.05	<0.01	0.01	0.20	0.14	0.06	0.14
Selenium	µg/L	0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Uranium	µg/L	0.001	<0.001	0.002	0.01	0.006	0.007	0.015	0.018	0.33	0.027
Zinc	µg/L	0.1	<0.1	<0.1	1.0	<0.1	<0.1	0.5	2.6	1.0	0.7
Calcium	mg/L	0.1	<0.1	<0.1	0.2	<0.1	<0.1	0.1	5.3	0.8	0.2
Magnesium	mg/L	0.1	<0.1	<0.1	0.9	<0.1	<0.1	0.6	<0.1	1.3	1.1
Sodium	mg/L	0.1	<0.1	<0.1	4.0	<0.1	<0.1	1.5	<0.1	<0.1	1.8
Sulphate	mg/L	0.5	<0.5	<0.5	100	<0.1	<0.1	98	0.3	6.3	97

Table S3.5: Water chemistry measurements of blanks, pro-blanks and control treatments for each test with Lake Bennett glochidia, and for two samples of Lake Bennett water.

Test			6. Lake Bennett (1586M)			7. Lake Bennett (1679M)			Lake Bennett water	
	Units	Detection limit	Blank	Pro-Blank	Control	Blank	Pro-Blank	Control		
Date sampled				6/5/17			6/5/17		9/2/17	22/11/17
Date analysed				29/5/17			29/5/17		22/2/17	7/12/17
Aluminium	µg/L	0.1	0.2	0.1	22	0.6	0.6	96	18	8.1
Cadmium	µg/L	0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Cobalt	µg/L	0.01	<0.01	<0.01	0.06	<0.01	<0.01	0.08	0.03	0.01
Chromium	µg/L	0.1	<0.1	<0.1	0.1	<0.1	<0.1	0.2	<0.1	<0.1
Copper	µg/L	0.01	0.03	0.06	0.12	0.03	0.06	0.17	0.62	1.3
Iron	µg/L	1	<1	<1	67	<1	<1	57	160	46
Manganese	µg/L	0.01	<0.01	<0.01	1.2	<0.01	<0.01	2.7	6.0	0.33
Nickel	µg/L	0.01	0.01	0.02	0.19	0.01	0.06	0.22	0.52	0.09
Lead	µg/L	0.01	<0.01	<0.01	0.1	<0.01	0.03	0.17	0.13	0.02
Selenium	µg/L	0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Uranium	µg/L	0.001	0.007	0.006	0.017	0.007	0.001	0.022	0.020	0.009
Zinc	µg/L	0.1	<0.1	<0.1	1.0	<0.1	<0.1	0.9	2.3	1.4
Calcium	mg/L	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	3.2	2.3
Magnesium	mg/L	0.1	<0.1	<0.1	0.4	<0.1	<0.1	0.2	2.0	1.8
Sodium	mg/L	0.1	<0.1	<0.1	2.2	<0.1	<0.1	2.9	3.7	4.3
Sulphate	mg/L	0.5	<0.5	<0.5	97	<0.5	<0.1	92	<0.5	<0.1

Table S3.6: Control survival of glochidia after 24-h using the different glochidia selection methods.

Glochidia selection method	Number of tests	Control survival (% ± SD)
Open glochidia	2	72.5 ± 8.2
Snapping	5	91.4 ± 9.5
Snapping / physical observations	12	98.8 ± 1.9

Table S3.7: Average measured pH, electrical conductivity and survival of glochidia after 24-h for method development tests with ammonia using the same protocol but different pH control methods.

Method of pH control	No. of tests	pH [a] (± SD)	pH Shift [b] (± SD)	EC of control (Mean, µS/cm)	EC Shift [b] (% shift, ± SD)	Dissolved Oxygen [a] (%, ± SD)	Control survival (%, ± SD)
None	6	6.3 ± 0.1	0.5 ± 0.1	16.3 ± 2.3	4.7 ± 3.5	92.7 ± 2.9	92.7 ± 12.4
CO_2	3	6.2 ± 0.1	0.1 ± 0.1	18.5 ± 1.8	5.0 ± 2.0	98.6 ± 3.2	81.7 ± 16.9
MES	1	5.9 ± 0.0	0.0 ± 0.0	49.0 ± 1.4	4.2 ± 2.0	98.3 ± 8.1	95.0 ± 0.0
HEPES	2	6.1 ± 0.0	0.1 ± 0.0	16.8 ± 0.4	8.8 ± 4.2	98.7 ± 0.9	95.8 ± 1.2

[a] None: n = 64; CO2: n = 40; MES: n = 4; HEPES: n = 12

[b] None: n = 32; CO2: n = 20; MES: n = 2; HEPES: n = 6

Table S3.8: Results of a triplicate 48-h test in Magela Creek water (MCW) to compare control survival using different test vessels and different handling methods (10 glochidia per treatment)

	Treatment	48-h Survival (%)
A	9cm Plastic petri dishes, 30mL MCW, minimal handling [a]	93
B	9cm Plastic petri dishes, 30mL MCW, extra handling [b]	89
C	12-well plate submerged in 500mL MCW, minimal handling	56
D	12-well plate submerged in 500mL MCW, extra handling	45

[a] Minimal handling = Glochidia were observed under the microscope in their test containers. A 2/3 water change was performed at 24-h by removing and replacing water, so that the glochidia were not handled.

[b] Extra handling = Glochidia were pipetted into a petri dish, swirled around, and observed under the microscope, then pipetted into new water during the 24-h water change.

Table S3.9: Acute ammonia toxicity data (mg/L TAN) comparisons from temperate freshwater mussel species showing the original LC_{50} reported, and adjustments made to pH 8 at 20°C, and pH 7 at 20°C. Where multiple test data were reported from the same species, the lowest values are shown.

Species name	**Location**	**LC50** (mg/L TAN) 24-h tests at various pH and temp in °C	**NH3 concentration** [a] (mg NH_3/L) At test conditions	**LC50** (mg/L TAN) Adjusted to pH 8, 20°C [b]	**LC50** (mg/L TAN) Adjusted to pH 7, 20°C [c]	**Reference**
Fatmucket *Lampsilis siliquoidea*	USA	7.3 (pH 8.3, 20°C) [d]	0.54	13.0	55.9	Wang *et al*. (2007a)
Ellipse *Venustaconcha ellipsiformis*	USA	5.0 (pH 8.3, 20°C)	0.37	8.9	38.3	Wang *et al*. (2007a)
Dwarf wedge mussel *Alasmidonta heterodon*	USA	>16 (pH 8.3, 20°C)	>1.17	28.5	122.5	Wang *et al*. (2007a)
Neosho mucket *Lampsilis rafinesqueana*	USA	8.3 (pH 8.3, 20°C)	0.61	14.8	63.5	Wang *et al*. (2007a)
Mucket *Actinonaias ligamentina*	USA	5.1 (pH 8.3, 20°C) [e]	0.37	10.2	43.6	Wang *et al*. (2007a)
Pink papershell *Potamiluso hiensis*	USA	>16 (pH 8.3, 20°C)	>1.17	28.5	122.5	Wang *et al*. (2007a)
Rainbow mussel *Villosa iris*	USA	12 (pH 8.3, 20°C)	0.88	21.4	91.9	Wang *et al*. (2007a)
Rainbow mussel *Villosa iris*	USA	0.284 mg NH_3/L (pH 8.1, 22°C)	0.28	6.1	26.2	Goudreau *et al*. (1993)
Rainbow mussel *Villosa iris*	USA	3.3 (pH 7.9, 20°C)	0.11	2.7	11.7	Scheller (1997)
Wavy-rayed lampmussel *Lampsilis fasciola*	USA	6.2 (pH 8.3, 20°C)	0.46	11.1	47.5	Wang *et al*. (2007a)
Paper pondshell *Utterbackia imbecillis*	USA	Not stated (pH 8, 25°C)	0.19	3.6	15.5	Black 2001 (in Augspurger 2003) [f, g]

Table S3.9: (Continued)…

Atlantic pigtoe *Fusconaia masoni*	USA	Not stated (pH 7.6, 25°C)	0.15	3.9	16.6	Black 2001 (in Augspurger 2003) [f]
kākahi *Echyridella menziesii (Hyriidae)*	New Zealand	9.6 (pH 7.8, 20.5°C)	0.24	6.9	29.8	Clearwater *et al*. (2014)
kākahi *Echyridella menziesii (Hyriidae)*	New Zealand	20.7 (pH 7.8, 20.5°C)	0.52	14.9	64.2	Clearwater *et al*. (2014)
kākahi *Echyridella menziesii (Hyriidae)*	New Zealand	21.9 (pH 7.8, 20.5°C)	0.55	15.8	67.9	Clearwater *et al*. (2014)
***Velesunio* sp.** *(Hyriidae)*	Australia	9.3 (pH 6.1, 27.3°C)	0.008	2.6	11.3	This study
Velesunio angasi *(Hyriidae)*	Australia	11.9 (pH 6.2, 27.6°C)	0.010	3.5	14.8	This study
Velesunio angasi *(Hyriidae)*	Australia	7.4 (pH 6.1, 27.3°C)	0.006	2.1	9.2	This study

[a] Data were adjusted using the speciation equations of Emerson *et al*. (1975)
[b] Data were adjusted to pH 8 and 20°C using algorithms from USEPA (1999).
[c] Data were adjusted to pH 7 and 20°C using algorithms from ANZECC/ARMCANZ (2000), and USEPA (1999).
[d] Data are lowest estimate from seven toxicity tests.
[e] Data are lowest estimate from four toxicity tests.
[f] Data have been re-adjusted to include temperature adjustments.
[g] Data are lowest estimate from three 48-h tests at the 24-h time point.

CHAPTER 4: Chronic ammonia toxicity to juveniles of 2 tropical Australian freshwater mussels (*Velesunio* spp.): Toxicity test optimisation and implications for water quality guideline values.

Abstract

Freshwater mussels play key roles in aquatic ecosystems, but are experiencing a global decline. Although studies have reported high acute sensitivity of mussels to some contaminants, chronic toxicity data are lacking for deriving high reliability water quality guideline values. Ammonia is a contaminant of potential concern in some catchments of tropical northern Australia, where freshwater mussels are important ecological and cultural species. The extremely soft waters (hardness <5 mg/L) of these environments can result in increased toxicity of many contaminants including ammonia, and regionally relevant tropical guideline values are needed to adequately protect these unique ecosystems. An optimised 14-d toxicity test protocol was used to assess the chronic toxicity of ammonia for juveniles of two species, the lotic *Velesunio* sp. and the lentic *Velesunio angasi*. Ammonia exposures were conducted at pH 6.0 and 27 ± 0.5 °C to represent local environmental conditions, using shell length growth rate as the endpoint. Chronic toxicity estimates indicated high sensitivity to ammonia, with mean median effect concentrations (in total ammonia nitrogen) being 7.0 mg/L for *V. angasi* from the semi-urbanised Lake Bennett, 9.2 mg/L for V. *angasi* from Sandy Billabong, and 11.3 mg/L for *Velesunio* sp. from Gulungul Creek. When the 10% effect concentration values were compared with other chronic ammonia data (normalised to pH 7.0 and 20 °C), *Velesunio* spp. were found to be more sensitive than 8 of 16 other temperate and 7 of 9 tropical invertebrate and fish species. These chronic toxicity estimates will be used to further inform regionally relevant and site-specific guideline values.

Keywords: Bivalves, Ecotoxicology, Mining, Aquatic invertebrates, *Velesunio angasi,* Water quality guidelines.

4.1 Introduction

Freshwater mussels play important roles in aquatic ecosystems, including biofiltration of the water column, nutrient and energy cycling, and sources of food for other animals and humans (Vaughn 2017). The global decline of freshwater mussel species is well documented, and is generally attributed to habitat loss, overexploitation, invasive species, and their high sensitivity to some contaminants (Lydeard *et al*. 2004; Augspurger *et al*. 2007; Lopes-Lima *et al*. 2017). Tropical waters with low ionic strength (hardness <5 mg/L) have been shown to increase the toxicity to tropical freshwater taxa of certain contaminants, such as metals (van Dam *et al* 2010; Harford *et al*. 2015; van Dam *et al*. 2017) and ammonia (Mooney *et al*. 2019). Mussels inhabiting these waters could also be at greater risk, but knowledge of their response to such contaminants is limited. Therefore, further research is needed to better understand impacts of contaminants on tropical mussel species, for the protection of these important taxa (Augspurger *et al*. 2007).

Although chronic toxicity data are preferred for generating high reliability water quality guideline values (Warne *et al.* 2018), the extrapolation of such values from short-term (acute) toxicity data is commonly practiced due to a lack of chronic data for many contaminants. (ANZECC/ARMCANZ 2000; Raimondo *et al*. 2007, May *et al*. 2016). A lack of chronic toxicity data for freshwater mussels has been attributed to the challenges researchers face when the mussels are used for longer-term toxicity testing. These challenges include difficulties culturing stocks in the laboratory, uncertainty regarding appropriate test durations and sub-lethal endpoints, and the need for including sediment in some juvenile mussel tests to sustain growth and survival (Augspurger *et al*. 2003; Newton and Bartsch 2007). Progress has been made in recent years through the development of specialised culturing and toxicity testing methods for freshwater mussel early life stages (Neves 2004; ASTM 2006; Barnhart 2006; Ingersoll *et al*. 2007). However, these methods were developed using temperate North American species (Unionidae), which may vary in culture requirements and toxicological sensitivity compared with species from other regions of the world.

Ammonia contamination in aquatic ecosystems is an important global issue, and reliable water quality guideline values or criteria are needed to protect local receiving waters (Camargo and Alonso 2006; Miao *et al.* 2013; USEPA 2013). Because freshwater mussels have a higher sensitivity to ammonia, it has been recommended that they be included when water quality guideline values or criteria are derived (Augspurger *et al*. 2003; Mummert *et al*. 2003; Wang *et al*. 2007a; USEPA 2013). However, most of the available ammonia toxicity

data for freshwater mussels are generated from temperate species (Augspurger 2003; US Environmental Protection Agency 2013, Clearwater 2014). In the US Environmental Protection Agency (USEPA) ammonia water quality criteria revision (USEPA 2013), temperate freshwater mussels were ranked the four most sensitive among 69 genera in all the acute data and the two most sensitive among 16 genera in all the chronic data. Studies have reported that although tropical species were more tolerant of some organic chemicals, they were generally more sensitive to ammonia than their temperate counterparts (Kwok *et al*. 2007; Wang and Leung 2015).

Ammonia is used for ore-processing at the Ranger uranium mine in tropical northern Australia, and has been identified as one of several contaminants of potential concern. Hence, site-specific toxicity studies that include the use of freshwater mussels are needed to determine the risk that ammonia presents to aquatic biota. Only limited acute ammonia data exist for some Australian tropical freshwater species, for example, barramundi, *Lates calcarifer*, the eastern rainbowfish, *Melanotaenia splendida splendida*, a freshwater shrimp, *Caridina nilotica* (Økelsrud 2004; Økelsrud and Pearson 2007), and two freshwater mussel species, *Velesunio angasi* and *Velesunio* sp. (Kleinhenz *et al*. 2018). Both *Velesunio* species were more sensitive to ammonia than most temperate species, as well as other tropical species, and were thus considered valuable additions to the data set for chronic ammonia toxicity. Chronic data were recently reported for the tropical green hydra, *Hydra viridissima* (Mooney *et al.* 2018), and for six tropical freshwater species (see Table 1.1, Mooney *et al.* 2019). At the time of the present study, no chronic ammonia toxicity estimates were available for tropical freshwater mussel species.

The aims of the present study were to: 1) optimise a standardised test protocol for the chronic exposure of tropical freshwater mussels to water-borne contaminants, and 2) use the test protocol to assess ammonia toxicity to the juvenile life stage of two tropical *Velesunio* species, thereby contributing to both the local and global chronic toxicity datasets for ammonia.

4.2 Materials and methods

The starting points for the toxicity test protocol used in the present study were the American Society for Testing and Materials International Standard (2006; ASTM E2455-06, Standard guide for conducting laboratory toxicity tests with freshwater mussels), and early work by Humphrey (1987a, 1987b, 1991), who successfully reared juvenile mussels of the same species (*V. angasi* and *Velesunio* sp.) in laboratory and field settings after exposing fish to the mussels' parasitic glochidial larvae.

4.2.1 Mussel species and collection

Several species of the genus *Velesunio* (Hyriidae family) are found throughout tropical northern Australia, the most common being *V. angasi* (Supplementary Data, Figure S4.1). Recent genetic analysis (J.M. Hughes, D.J. Schmidt, Griffith University, QLD, Australia, unpublished data) has confirmed the presence of *V. angasi*, a lentic species predominantly found in permanent billabongs, lakes and impoundments, and has identified a lotic species of *Velesunio* (*Velesunio* sp.), inhabiting creeks and streams that commonly cease to flow during the dry season (April–November). Adult *Velesunio* sp. and *V. angasi* were collected from three locations within the Alligator Rivers Region of the Northern Territory, Australia, Magela Creek (latitude 12°40'28''S, longitude 132°55'52''E), Gulungul Creek (latitude 12°39'21''S, longitude 132°52'42''E), and Sandy Billabong (latitude 12° 54' 4''S, longitude 132° 46' 38''E), and one location outside of the Alligator Rivers Region, the semi-urbanised Lake Bennett located 80 km south of Darwin (latitude 12° 57' 39'', longitude 131° 09' 59''). *Velesunio* sp. was sourced from the Magela Creek and Gulungul Creek sites, and *V. angasi* from Sandy Billabong and Lake Bennett. Mussels were transported back to the laboratory at ambient temperature (~28-32°C) within 4 h of collection, in aerated plastic 20-L drums containing approximately 15 L of water collected from the sampling site.

4.2.2 Glochidia isolation

Glochidia were isolated from female mussels according to the passive release method described in Kleinhenz *et al.* (2018). Viability testing was undertaken on a subsample of mature glochidia by exposure to a concentrated salt solution according to the method detailed in ASTM (2006). Only glochidia that had achieved >80% viability (preferably >90%) were used for host fish exposure, as recommended in ASTM (2006). The sub-samples were then discarded, and the remaining glochidia were pooled into a 400-mL watch-glass and gradually

acclimated to filtered Darwin tap water at a temperature of approximately 26 to 27°C for at least 3 h, to prepare for the host fish exposure in filtered Darwin tap water.

4.2.3 Host fish exposure and selection of juveniles

The northern trout gudgeon, *Mogurnda mogurnda*, was used as a host fish for all toxicity tests. This species is common in the Alligator Rivers Region, and has previously been identified as a host fish for *Velesunio* spp. (Humphrey and Simpson 1985). For the host-fish exposure, two *M. mogurnda* of approximately 12 cm length (~6 month old) were exposed to glochidia from the pooled sample for 35 min, in 4 L of vigorously aerated filtered Darwin tap water at a final density of approximately 19 000 glochidia/L. Exposed fish were transferred to a plastic tub containing approximately 60 L of filtered Darwin tap water, and held for up to 10 d until the required number of juvenile mussels had been collected. Fish were fed Hikari® sinking carnivore pellets (Kyorin) and one-third of the water was exchanged daily.

Excysted juveniles (measuring ~280 µm length, ~230 µm width) were collected by siphoning the material in the bottom of the tub through a 63-µm sieve. Collected juveniles were acclimated to the test diluent (Magela Creek water) for a minimum of 3 h, and used in toxicity tests within 24 h of being isolated from fish. The development of the host-fish exposure method is described in further detail in Appendix B.

Juveniles were selected under a stereomicroscope (Leica MZ8), and transferred into test vessels using a 1-mm-diameter Pasteur pipette. Criteria for selection of juveniles included opaque appearance, the presence of internal organs observed through the part-translucent shell and pedal gape, and a moving foot. During all tests, vessels were arranged randomly on Perspex trays and placed into constant-temperature incubators (27.5 ± 1 °C; Labec).

4.2.4 General laboratory procedures

All plastic and glassware used throughout experiments were cleaned by soaking in a 5% (v/v) nitric acid (HNO_3) bath for 24 h, triple rinsing in deionised water (Elix, Millipore), and then washing in a laboratory dishwasher (Miele) using deionised water and a phosphate-free detergent (Neodisher® Laboclean; Dr.Weigert).

4.2.5 Test diluent

The test diluent, Magela Creek water, is representative of the extremely soft waters of northern Australia. These waters are slightly acidic (pH 5.5–6.5), have low ionic strength (hardness 3–6 mg/L as $CaCO_3$), low alkalinity (5–10 mg/L as $CaCO_3$), and low electrical

conductivity (5–20 µS/cm; van Dam *et al.* 2010). Values for key physicochemical variables associated with the Magela Creek water used for definitive ammonia tests during the present study are presented in Table 4.1. The Magela Creek water was collected monthly from a permanent water body (Bowerbird Billabong, latitude 12° 46’ 15’’, longitude 133° 02’ 20’’). Water was pumped into 20-L acid-washed plastic containers and transported at ambient temperature to the laboratory within 4 h of collection, and was then filtered (3 µm Sartopure PP2 MidiCap filter; Sartorius Stedim) and stored at 4 °C until required for use.

Table 4.1: Mean physico-chemical composition of 0.45 µm filtered Magela Creek water (MCW) used in definitive ammonia tests (n = 6). Values represent the mean ± standard deviation of control treatment samples taken from each test.

Physicochemical variable	**MCW**	
pH	6.02 ± 0.03	
Conductivity (µS/cm)	14.9 ± 1.9	
Dissolved oxygen (%)	99.1 ± 1.6	
Alkalinity (mg/L as $CaCO_3$)	2.4 ± 1.5	
Hardness (mg/L as $CaCO_3$)	2.5 ± 0.4	
DOC (mg/L)	2.3 ± 0.8	
Analyte	**MCW**	**Detection limit**
Al (µg/L)	29.5 ± 9.4	0.1
Cd (µg/L)	<0.02 ± 0.0	0.02
Co (µg/L)	0.16 ± 0.04	0.01
Cr (µg/L)	0.17 ± 0.08	0.1
Cu (µg/L)	0.18 ± 0.07	0.01
Fe (µg/L)	120 ± 66	1
Mn (µg/L)	8.5 ± 1.5	0.01
Ni (µg/L)	0.16 ± 0.06	0.01
Pb (µg/L)	0.04 ± 0.01	0.01
Se (µg/L)	<0.2 ± 0.0	0.2
U (µg/L)	0.03 ± 0.01	0.001
Zn (µg/L)	0.44 ± 0.13	0.1
Ca (mg/L)	0.2 ± 0.1	0.1
Mg (mg/L)	0.5 ± 0.9	0.1
Na (mg/ L)	2.1 ± 0.5	0.1
Sulphate, SO_4 (mg/L)	92.5 ± 7.2	0.5

4.2.6 Toxicity test optimisation

The 14-d toxicity test optimisation experiments involved trialling different variables, including test species, test volumes, test vessels, the addition of fine sediment, food and feeding frequency, pH control, and biological endpoints. For these experiments, control performance and endpoint responses were assumed to be comparable among the mussel species and populations used. One-way and two-way analysis of variance (ANOVA) tests or Student's *t*-tests were used (α = 0.05; SigmaPlot 13.0) to compare test optimisation results for selected treatments. The optimisation experimental methods are described in detail in Appendix C.

4.2.7 Toxicity testing

The final toxicity testing protocol (Table 4.2) was based on the results of the test optimisation experiments. Using the optimised protocol, six definitive chronic ammonia tests were undertaken (two tests with *Velesunio* sp. from Gulungul Creek, two tests with *V. angasi* from Sandy Billabong, and two tests with *V. angasi* from Lake Bennett) to estimate and compare ammonia toxicity between juvenile mussels from different sites and species. Test duration was set at 14 d, the minimum test period required to classify the test as chronic, using the guidance of Warne *et al*. (2018). Other chronic toxicity studies with temperate mussel species have used a 28-d test duration (Wang *et al*. 2007c, Wang *et al*. 2011) as recommended in ASTM (2006), but Warne *et al*. (2018) note that test durations may be reduced for tropical species whose life cycles are much shorter than those of temperate species.

The growth rate endpoint was determined using image analysis software (Leica application suite, Ver 4.6.1) to measure the maximum shell length of each mussel photographed on days 0 and 14 (Leica MC170HD camera, Leica M205C microscope at 8.0× magnification).

4.2.7.1 Preparation of test solutions

Analytical grade ammonium sulfate $(NH_4)_2SO_4$ (Sigma-Aldrich) was added to high-purity water (MilliQ; Millipore) to prepare a 1000-mg/L total ammonia nitrogen (TAN) stock solution. A bulk batch of Magela Creek water and sediment was prepared to achieve a diluent with a turbidity of 100 nephelometric turbidity units (NTUs; TPS 90-FLT meter, probe

125186). Each test concentration was prepared by mixing the diluent with the required volume of ammonia stock solution and 1 mM of N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) buffer. The initial pH was adjusted to 6.0 ± 0.1 by adding 5% H_2SO_4 or 5% NaOH dropwise.

Table 4.2: The final 14-d chronic toxicity test design

Test organism	
Species	*Velesunio* spp.
Life stage	Juvenile <24-h-old post excystment
Host-fish exposure method for acquiring juvenile mussels	
Host fish	2 × *Mogurnda mogurnda* juveniles ~15 cm
Exposure volume	4 L tap water in 5-L beaker, aerated with air stone
Exposure duration	35 min, agitated every 10 min with air stone
Glochidia quantity	Medium (~75,000 glochidia, or ~18/mL)
Toxicity test description	
Type	Static renewal
Test duration	14 d
Test vessels	6-cm plastic jars (175 mL) with lids
Test volume	100 mL
No. of organisms per test vessel	10
No. of replicates per concentration	2
Dilution water	Natural Magela Creek water
Test solution renewal	100% every 2 d
Feeding	*Chlorella* algae (~7.0–8.0 × 10^4 cells/mL) every 2 d
Aeration	None
Turbidity at start and at water changes	100 NTU using fine sediment ≤63 µm
Assessment endpoint	Growth rate, survival
Quality Control	
Test acceptability	Survival >80% in each control treatment
Temperature	27.5 ± 1°C
pH	6.0 ± 0.3
pH control	1 mM HEPES buffer adjusted to pH 6.0
Dissolved oxygen	80–120% saturation
Photoperiod	12:12-h (light: dark)

NTU = nephelometric turbidity unit; HEPES = N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid.

Test vessels were prepared by adding the required volume of algae (*Chlorella* sp.), and then dispensing 100 mL of each test concentration into the vessels to ensure thorough mixing. Dispensed test vessels and water samples were warmed to the testing temperature, and the pH further adjusted if necessary, by adding 1% H_2SO_4 or 1% NaOH dropwise to ensure the starting pH was 6.0 ± 0.1.

4.2.7.2 Quality control

Test waters from each treatment were measured for water quality using a WTW Multi 340i meter, with specific probes: Sentix 41 for pH, Orion 013005MD for electrical conductivity and CellOx 325 for dissolved oxygen. New waters were measured at the start of each test and during each water change, and pooled replicate subsamples from old waters were measured during each water change and at the end of the test. For each treatment, measurements taken over the test duration were averaged, and were reported and used in further analyses of ammonia toxicity. Incubator temperature was monitored at 5-min intervals using data loggers (Testo Saveris™) throughout each test. Care was taken during water changes to ensure that water temperatures of the test containers did not change significantly while out of the incubator, by using heat pads set at an appropriate temperature. New test waters were acclimated to the required temperature for 2-3 hours prior to test commencement and water changes.

Total organic carbon (TOC) and dissolved organic carbon (0.45 µm filtered) for each batch of Magela Creek water were measured in-house (Shimadzu TOC-V CSH device) within 48 h of collection.

Blank, procedural blank, and diluent water samples from each test were filtered (0.45µm, Minisart RC25 filter; Sartorius Stedim), acidified using 1% HNO_3, and sent to an external laboratory (Envirolab, Chatswood, NSW, Australia) for chemical analysis of a standard suite of metals and major ions (Al, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Na, Ni, Pb, Se, SO_4, U and Zn) using inductively coupled plasma-mass spectrometry and inductively coupled plasma-atomic emission spectroscopy. Ammonia concentrations (as TAN) of unfiltered subsamples were measured at the start and end of tests using an ammonia test kit (Palintest), followed by spectrophotometry (Shimadzu UV-2550, wavelength 640 nm; USEPA method 350.1; USEPA 1993), and were averaged for concentration-response analysis.

Tests were considered valid if control growth was ≥ 12.8 µm/d (see the *Toxicity test optimisation* section in the *Results*), control survival was ≥80% at the end of the test (ASTM 2006), average changes in electrical conductivity remained within 10% of the values at test commencement, drift in pH did not exceed ±0.3 units from the starting pH in a 48-h period, dissolved oxygen levels remained >80%, and temperature of the incubator remained within ±1 °C of the target.

4.2.7.3 Data analysis

Measured TAN concentrations were used for concentration-response modelling and estimation of effect concentrations (EC*X*) values. Growth rate data for each definitive ammonia toxicity test were transformed to percentage of control, and the data from each of the two tests/site were pooled to generate toxicity estimates for the three sites and two mussel species (*Velesunio* sp.: Gulungul Creek, *V. angasi*: Sandy Billabong and Lake Bennett) using CETIS™ (Ver 1.9.0.9; Tidepool Scientific) for statistical analysis. The toxicity estimates were calculated from concentration-response curves generated using non-linear (3-parameter logistic) regression. Ammonia toxicity estimates were calculated for a 10% (EC10), 20% (EC20) and 50% (EC50) reduction in growth relative to the control responses, along with their 95% confidence limits. For comparisons with other chronic ammonia data, EC*X* values were adjusted to pH 7 or 8 and 20°C using the algorithms derived in Emerson *et al*. (1975) and described in the USEPA method (USEPA 2013). See Appendix D for further information about each method.

4.3 Results

4.3.1 Toxicity test optimisation

4.3.1.1 Test volume and test vessels

All four preliminary trials comparing growth and survival among different test volumes and test vessels showed consistently best performance in 100-mL treatments in plastic jars with lids (Supplementary Data, Figure S4.2). In a test comparing two feeding regimes (*Chlorella* sp. and shellfish diet, both at ~8 x 10^4 cells/mL), growth was almost doubled in 100-mL treatments compared with the 30-mL treatments regardless of feeding regime (Supplementary Data, Figure S4.2a). In two tests comparing 100 with 50 mL (Supplementary Data, Figure S4.2b and c) and one test comparing 100 with 150 mL (Supplementary Data, Figure S4.2d), growth was higher in the 100-mL treatments, whereas survival was 100% in all treatments. In the final growth trial, the 100-mL treatments also achieved the greatest growth rate (12.8 µm/d), with significant differences found between treatments (P = 0.007; Figure 4.1). Survival rates remained within acceptable levels (≥80%), ranging from 83% (125-mL treatment) to 97% (150-mL treatment).

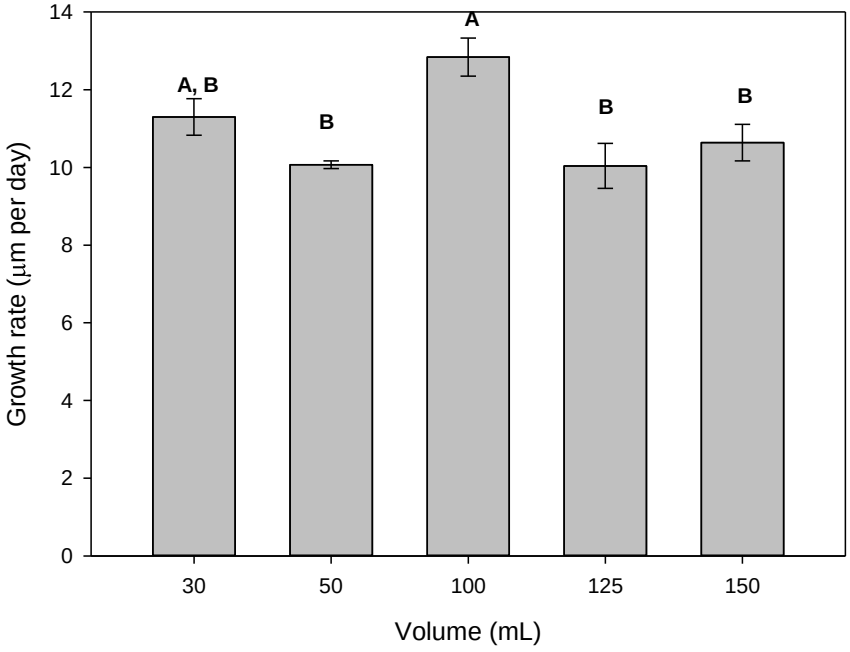

Figure 4.1: Results of a 14-d test comparing growth rates (± standard error of 3 replicates) of juvenile mussels from Lake Bennett (*Velesunio angasi)* in different test volumes. All tests were carried out in Magela Creek Water with algae (*Chlorella* ~8.0 × 10^4 cells/mL) in 175 mL jars (6-cm diameter) with lids. Initial turbidity was 100 nephelometric turbidity units (NTUs). Survival rates for 30-, 50-, 100-, 125-, and 150-mL treatments were 90, 93.3, 93.3, 83.3, and 96.7% respectively. Significant differences were found between treatments (one-way analysis of variance, $p = 0.007$). Different capital letters denote significantly different treatments, as detected by pairwise multiple comparison (Holm-Sidak method).

4.3.1.2 Sediment and feeding

In turbidity trials, growth rates were highest in treatments with the highest turbidity (100 and 200 NTU), regardless of feeding regime. Growth was significantly reduced in treatments with no sediment added (Supplementary Data, Table S4.1: tests 2 and 3). Results of the final combined feeding and turbidity trial are described below.

In feeding trials, **s**hellfish diet or *Chlorella* sp. supplemented with FFV (Fermented Food with Vitamins, see Appendix C: Sediment and feeding), produced higher growth than algae alone, but survival was reduced (Supplementary Data, Table S4.2: tests 1 and 2), and fungal growth was increased on the mussel shell surface. The optimum cell density for shellfish diet was 8 x 10^4 cells/mL (Supplementary Data, Figure S4.3). The *Chlorella* sp. diet resulted in higher growth rate than the shellfish diet (Supplementary Data, Table S4.2; tests 3 and 4), and

mussels were healthier, with sediment and algae tending to adhere to the mussel shell surface in treatments using the shellfish diet (Supplementary Data, Figure S4.4).

In the final combined feeding and turbidity trial (Figure 4.2), significant differences in growth rates were observed among turbidity treatments (p = 0.005), with 200 NTU producing the greatest growth. Significant differences were also observed among algal treatments (p = 0.01), and greatest growth was observed in treatments using 7.6 x 10^4 cells/mL *Chlorella* sp. in all turbidities. Two treatments with 100 and 200 NTU using this algal cell count produced the greatest growth (31.5 and 32.6 µm/d, respectively), with no significant difference (p = 0.23, one-way ANOVA) found between these 2 treatments. Therefore, the smallest possible amount of added sediment that produced no significant difference in growth compared with the next highest sediment concentration tested was chosen (100 NTU) to minimise factors that may modify ammonia toxicity and to simplify transfer of juveniles during water changes. On the basis of these results, approximately 7.0 – 8.0 x 10^4 cells/mL *Chlorella* sp. and 100 NTU were used in the final test protocol.

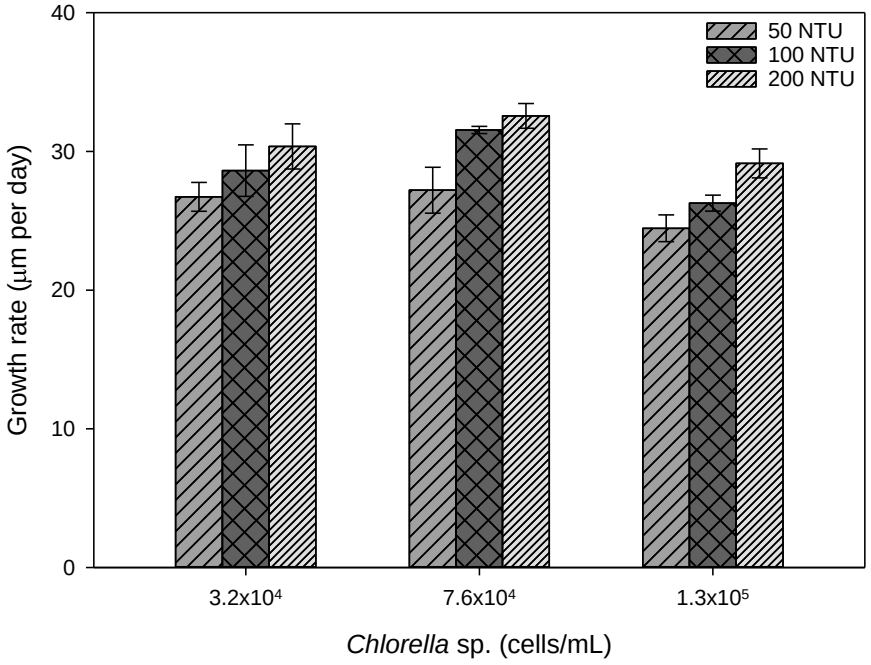

Figure 4.2: Mean growth rate of juvenile *Velesunio angasi* fed 3 different volumes of Chlorella sp. at 3 different turbidities. Columns represent the mean of 3 replicates ± standard error. Significant differences were seen among algal treatments (p = 0.010) and turbidity treatments (p = 0.005) using 2-way analysis of variance. Pairwise multiple comparison (Holm-Sidak method) detected significant differences (P < 0.05) among all turbidity treatments, and among all Chlorella sp. treatments except those with 3.2 x 10^4 cells/mL and 1.3 x 10^5 cells/mL (P = 0.075). NTU = nephelometric turbidity units.

4.3.1.3 Water changes and pH control

Growth was reduced when water was changed less frequently than every 2 d, with growth and survival lowest when water was changed every seventh day (Supplementary Data, Table S4.3). In the absence of added toxicants and buffers, water quality remained acceptable in all treatments: (pH 6.2 - 6.4, electrical conductivity 13.0–13.5 µS/cm, dissolved oxygen 98.9–99.3 %). Based on these results, water changes every 2 d were undertaken in subsequent toxicity testing. Both the HEPES and the 2(N-morpholino) ethanesulfonic acid (MES) buffers controlled pH within acceptable levels (6.0–6.1; Supplementary Data, Table S4.4). When either buffer was used, no significant difference (p = 0.24) was seen in median growth (HEPES 31.9 and MES 33.5 µm/d), and survival remained at ≥95%. Both buffer treatments showed an increase in electrical conductivity compared with Magela Creek water (~14 µS/cm), with the treatments using MES measuring a higher average electrical conductivity (59.4 µS/cm) than the HEPES treatments (25.9 µS/cm), and thus HEPES was chosen as the preferred buffer.

4.3.1.4 Endpoints

Dry weight measurements were more variable than shell growth rate measurements (Supplementary Data, Figures S4.5 and S4.6), and the two endpoints had a low correlation in both tests (r^2 = 0.468 and 0.065, respectively; Supplementary Data, Figures S4.7 and S4.8). On this basis, dry weight was discontinued as a viable and useful endpoint. Survival data recorded during tests were used for quality control, and to optimise concentration ranges to minimise mortality. Control growth rates measured during optimisation tests averaged 21.2 ± 5.8 µm/d (± standard deviation [SD]), ranging from 12.8 to 31.9 µm/d with various feeding and turbidity regimes. The minimum growth rate in these tests (12.8 µm/d) was used for the control growth test acceptability criterion for toxicity tests.

4.3.2 Toxicity tests

4.3.2.1 Quality control

For all ammonia toxicity tests, control survival met test acceptability criteria, averaging 98.3 ± 2.6% (± SD). Control growth rates averaged 25.0 ± 4.6 µm/d (± SD), also meeting the test acceptability criterion. Mean physicochemical variables (± SD) remained within acceptability criteria in all tests with pH 6.01 ± 0.02, dissolved oxygen 100.6% ± 1.2% saturation and temperature 27.2 ± 0.2°C (Table 4.3).

Table 4.3: Mean physicochemical variables of test solutions across all definitive ammonia tests (± standard deviation).

Test	Date	Species	Site	Control survival (%)	Control growth (µm/day)	pH (Units) [a]	Temp. (°C) [b]	EC (µS/cm) [a]	EC shift (%) [a]	Dissolved Oxygen (%) [a]
1	12/03/16	*Velesunio* sp.	GC	95	32.7 ± 0.8	6.00 ± 0.05	26.9	139.1	1.2 ± 0.7	100.2 ± 2.7
2	29/04/17	*Velesunio* sp.	GC	100	19.9 ± 0.5	5.97 ± 0.04	27.4	68.7	1.1 ± 1.3	100.7 ± 2.5
3	05/06/17	*Velesunio angasi*	SB	100	22.9 ± 0.2	6.03 ± 0.06	27.2	77.5	2.5 ± 2.1	102.3 ± 3.1
4	25/06/17	*Velesunio angasi*	SB	95	21.6 ± 0.8	6.00 ± 0.05	27.2	104.7	2.2 ± 1.1	98.8 ± 3.4
5	23/03/17	*Velesunio angasi*	LB	100	30.7 ± 0.4	6.02 ± 0.04	26.9	117.5	1.1 ± 1.3	100.6 ± 2.8
6	13/05/17	*Velesunio angasi*	LB	100	21.0 ± 1.3	6.01 ± 0.05	27.4	71.7	1.5 ± 1.9	101.2 ± 2.5
		Mean ± SD		*98.3 ± 2.6*	*24.8 ± 5.5*	*6.01 ± 0.02*	*27.2 ± 0.2*	*96.5 ± 28.5*	*1.6 ± 0.6*	*100.6 ± 1.2*

[a] Values represent measurements of test waters at 0 h and 2 replicate samples at 2, 4, 6, 8, 10, 12, and 14 d from all treatments from each test (tests 2 and 6: $n = 7$; test 1: $n = 8$; tests 3–5: $n = 9$).

[b] Incubator temperature was monitored at 5-min intervals using data loggers (Testo Saveris™). New test waters were acclimated for 2-3 hours prior to test commencement and water changes, and heat pads were used during transfer of juveniles into new water.

EC = electrical conductivity; GC = Gulungul Creek; SB = Sandy Billabong; LB = Lake Bennett.

Mean electrical conductivity of the control treatment using Magela Creek water was 14.9 µS/cm ± 1.9 (Table 4.1). The mean electrical conductivity among ammonia treatments (1.4–31.1 mg/L TAN) ranged from 19 to 345 µS/cm.

The analyses of blank and procedural blank samples from the start of each test showed that no confounding contamination was present. Measured chemical concentrations of the control treatments (Magela Creek water containing algae and sediment) at the start of each test reflected normal Magela Creek water composition. Measured ammonia concentrations generally remained within 10% between the start and the end of tests, averaging a net decrease of 3.9% for the duration of the tests (Supplementary Data, Table S4.5).

4.3.2.2 Toxicity of ammonia

The toxicity of ammonia to mussels among the three different field sites was notably different (Figure 4.3 and Table 4.4). The toxicity estimates for *Velesunio* sp. from Gulungul Creek were the highest (EC50 = 11.3, EC20 = 5.6, EC10 = 3.7 mg/L TAN), whereas those of *V. angasi* from Lake Bennett were the lowest (EC50 = 7.0, EC20 = 4.1, EC10 = 2.9 mg/L TAN). The EC50 (9.2 mg/L) and EC20 (4.4 mg/L) values for *V. angasi* from Sandy Billabong were between the values of those found at the other two sites, whereas the EC10 value (2.8 mg/L) was comparable to that of *V. angasi* from Lake Bennett. Based on the EC50 and EC20 values from each site (at test pH and temperature), the order of sensitivity was Lake Bennett > Sandy Billabong > Gulungul Creek. Based on EC10 values, the order of sensitivity was Sandy Billabong > Lake Bennett > Gulungul Creek. The mean EC50s among the sites varied by a factor of 1.6, the mean EC20s by 1.4, and the mean EC10s by 1.3.

When the EC10 values for *Velesunio* spp. were adjusted to pH 7 and 20°C using the USEPA method (USEPA 2013), and compared with chronic data (EC10 or no-observed-effect concentration [NOEC] values) for 25 other species belonging to 12 taxa, *Velesunio* spp. were more sensitive than 8 of 16 temperate species, and more sensitive than 7 of 9 tropical species (Figure 4.4 and Supplementary Data, Table S4.6).

Using only the equations of Emerson *et al.* (1975) for adjusting data, *Velesunio* spp. were found to be more sensitive than all 16 temperate species, and all except one tropical species (*H. viridissima*); Supplementary Data, Figure S4.9 and Table S4.6).

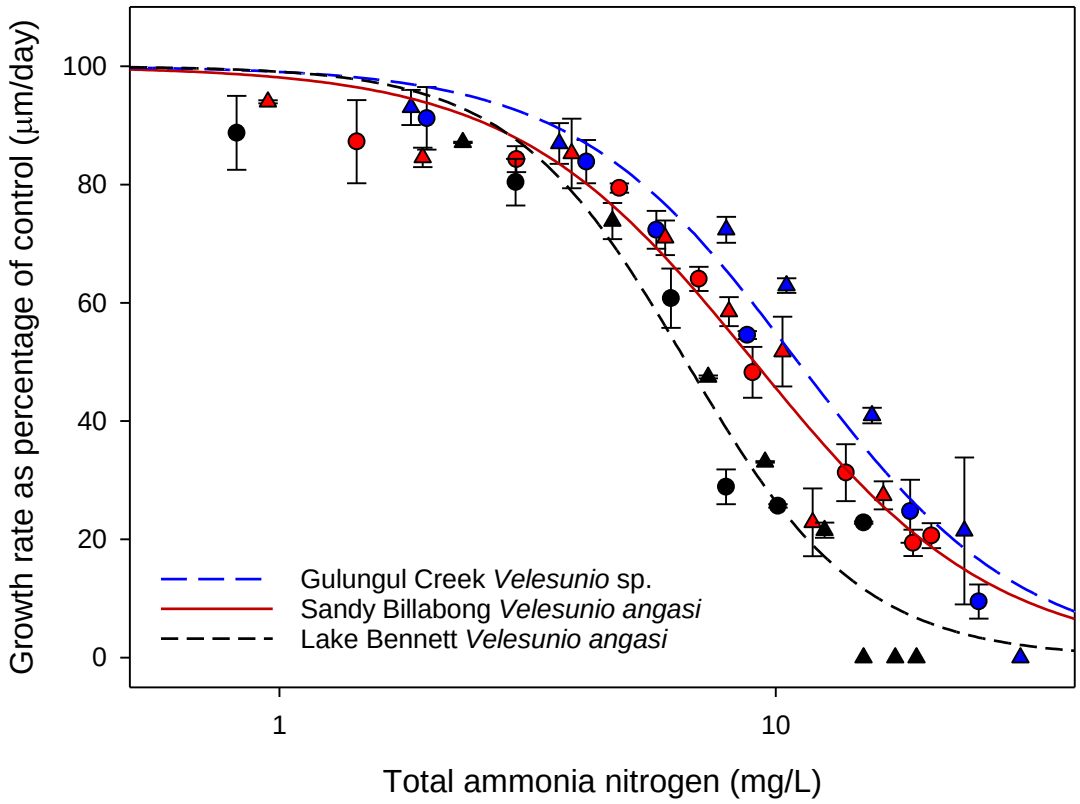

Figure 4.3: Comparison of the ammonia concentration-response relationships for 14-d exposures between *Velesunio* sp. from Gulungul Creek, and *Velesunio angasi* from Sandy Billabong and Lake Bennett. Data points represent the mean ± standard error of two replicates/ treatment. Symbols represent the first (triangle) and second (circle) toxicity tests. Toxicity estimates were determined using 3-parameter logistic models.

Table 4.4: Summary of the mean pH, temperature and ammonia toxicity estimates for pooled tests with *Velesunio* spp. [a]

				NH3 (mg/L)	Total Ammonia Nitrogen (mg/L)		
Species	Site	pH (SD)	Temperature (°C)	EC50	EC50 (95% CL)	EC20 (95% CL)	EC10 (95% CL)
Velesunio sp.	Gulungul Creek	5.99 (0.02)	27.2	0.007	11.27 (9.75 – 13.03)	5.63 (4.24 – 6.93)	3.68 (1.91 – 4.98)
V. angasi	Sandy Billabong	6.02 (0.02)	27.2	0.006	9.18 (8.21 – 10.28)	4.38 (3.43 – 5.30)	2.78 (1.62 – 3.71)
V. angasi	Lake Bennett	6.02 (0.01)	27.2	0.005	7.00 (6.18 – 7.93)	4.06 (2.97 – 4.98)	2.90 (1.09 – 3.87)

[a] Two pooled tests from each site; Gulungul Creek: $n = 15$; Sandy Billabong: $n = 18$; Lake Bennett: $n = 16$).

SD = standard deviation; EC = effect concentration; CL = confidence limit.

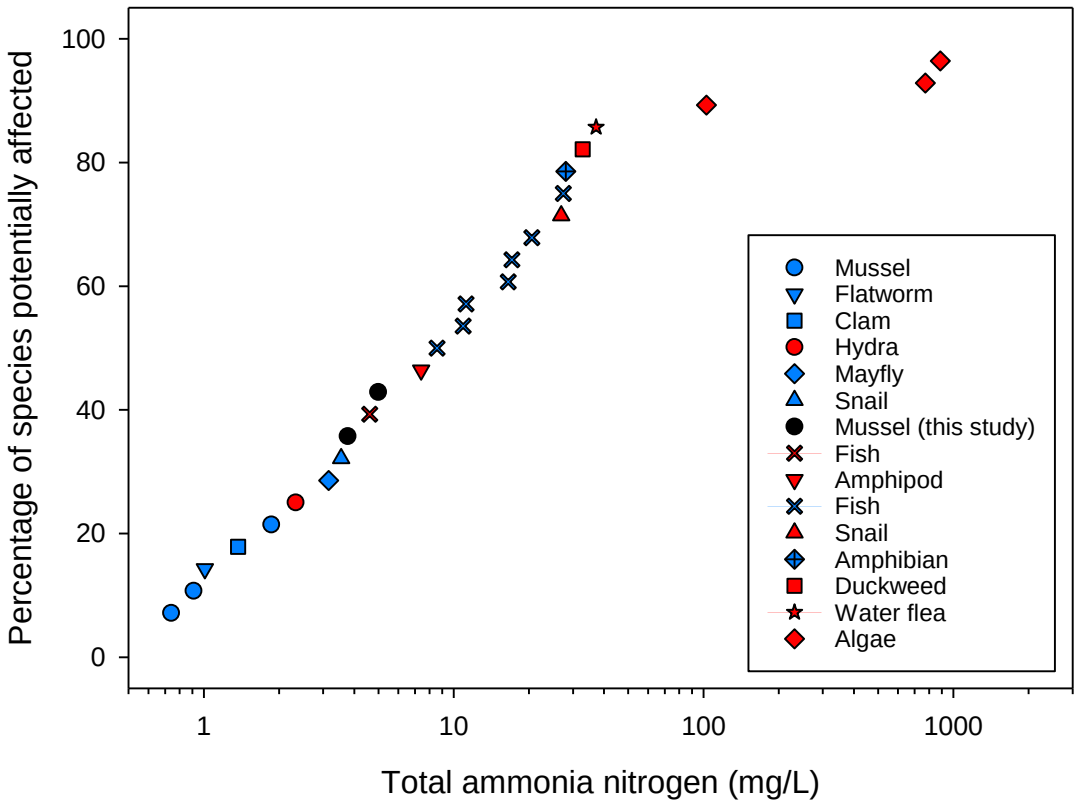

Figure 4.4: Comparison of the sensitivity of *Velesunio* spp. ammonia data (effect concentration, 10% [EC10]) from the present study (black circles) with available chronic ammonia data (EC10/no-observed-effect concentration) from temperate freshwater taxa (blue symbols) and tropical freshwater taxa (red symbols). Data were adjusted to pH 7 and 20 °C using the USEPA (2013) adjustment method. Original values at test conditions are shown in the Supplementary Data, Table S4.6.

4.4 Discussion

4.4.1 Toxicity test optimisation

4.4.1.1 Test volume and test vessels

A wide range of test volumes have been used in juvenile mussel studies (ASTM 2006), varying from 3.5 mL/replicate in static conditions to 1200 mL/replicate in flow-through conditions. Similarly, many different test vessels have been trialled, including Petri dishes, 12-well plates, crystallising dishes, and beakers (ASTM 2006). In the present study, the optimal test volume and vessel for the 14-d test protocol using 10 juveniles/vessel was 100 mL in a 175-mL plastic jar. No significant difference was observed between 100- and 150-mL volumes (p = 0.35), which further supported the use of 100-mL volumes. The water volume and depth appeared to be important factors influencing mussel growth, with the lowest growth rate observed in shallow, small-volume Petri dishes. In mussel culturing, static culture systems using small volumes have been effective in sustaining juvenile mussels in their early pedal-feeding stage (Jones and Neves 2002). However, using low volumes of water can be a disadvantage due to the greater risk of fungal contamination, which could reduce growth and survival (Eybe *et al.* 2013).

4.4.1.2 Addition of sediment

The addition of sediment proved necessary for achieving optimal mussel growth. Juvenile mussels feed on a mixture of fine particulate organic matter including detritus, bacteria and algae (Cope *et al*. 2008). Sediment as a suspension or layer has been reported to support juvenile mussel growth and survival (Gatenby *et al.* 1996), by facilitating the collection of food using pedal-feeding (Gatenby *et al*. 1996; Hua *et al*. 2013), providing a nutritional resource, and facilitating digestion and feeding orientation (Jones *et al*. 2005). When testing with *Velesunio* spp., Humphrey (1987a, 1987b) applied the early North American culturing methods of Hudson and Isom (1984), who found that the addition of a silt suspension in test vessels increased growth and survival of post-excysted juveniles.

Different sediment particle sizes were not trialled during the present study, because the size of ≤63 µm proved to be appropriate for producing the required turbidity and subsequent high mussel survival and growth. Fine sediments of different size classes (45, 45-63, and 63-125 µm) and concentrations (0-10 g/L) have been shown to have no negative effects on mussel filtration (Lummer *et al*. 2016). Growth of *Lampsilis siliquoidea* (Unionidae) has

been positively related to volatile solids, total phosphorus and particular fatty acids in sediment, and water particle size fractions <32 µm, and negatively related to fatty acids in the >63 µm fraction (Bartsch *et al*. 2017).

Studies have also shown that using sediment or detritus in combination with algal food produces higher growth and survival rates for juvenile mussels (Gatenby *et al.* 1996, 1997; Eybe *et al.* 2013). Gatenby *et al.* (1996) observed more algae in the juvenile gut of the rainbow mussel (*Villosa iris*) when they were reared on sediment and algae than those reared on algae alone. They concluded that fine sediment provided nutritional value and facilitated collection of food materials by pedal-feeding juvenile mussels, and suggested that fine sediment may aid in the digestion of algal cells by acting as an internal grinding substrate.

Although ammonia does not bind to sediment, using large amounts of sediment in ammonia toxicity tests can be challenging because of the tendency for partitioned ammonia to diffuse from sediment porewater into the overlying water. This may increase pH levels or cause a pH gradient in overlying water (Newton and Bartsch 2007). However, the small amount of sediment added to treatments during this study produced only a thin covering on the bottom of test vessels after settling, minimising potential for pH gradients. Wang *et al.* (2011) observed similar concentration-response relationships to ammonia in treatments with or without sediment, despite higher growth rates and survival in sediment treatments, indicating that sediment did not substantially influence mussel sensitivity to ammonia. Minimising the amount of sediment used in the toxicity test would be more important if the protocol is used to assess metals, due to the potential of metals to bind to the sediment. Sediment-bound metals may become biologically unavailable to the mussels, reducing toxicity (Costello and Burton 2014; Cardoso-Silva *et al*. 2016).

4.4.1.3 Feeding

The most appropriate algal diet for juvenile *Velesunio* spp. during toxicity testing was *Chlorella* sp. The improved growth and survival of juveniles using the *Chlorella* sp. diet, compared with using the mixture of algae present in the shellfish diet, may be due to its smaller cell size and higher nutritional content. The diet of *V. angasi* was previously investigated by Humphrey and Simpson (1985), who observed that *V. angasi* in Magela Creek were phytophagous and detritivorous filter feeders, with their main food comprising unicellular algae and organic detritus. Unicellular algae were considered to be the main useable energy source rather than organic detritus or diatoms. Laboratory-cultured green algae, and commercially available (nonviable) marine algae such as in the shellfish diet, have

been used successfully by other researchers for temperate freshwater juvenile mussel propagation and toxicity testing (Gatenby *et al.* 1996; Ingersoll *et al*. 2007; Wang *et al*. 2007b; Wang *et al.* 2007c; Eybe *et al.* 2013).

The increased growth measured in treatments with FFV may have been due to its greater nutritional value but may also have been a result of the reduced survival and therefore less competition for food for remaining mussels. The reduced survival was likely to be a result of the FFV introducing unknown bacteria or fungi, which grew on mussel shells and agglomerated sediment particles (Supplementary Data, Figure S4.4). This growth around the valve opening may have affected their ability to pedal-feed. Jones *et al*. (2005) recommended careful preparation of sediment before use, including autoclaving to kill invertebrate predators that may induce mortality by consuming or encasing juvenile mussels. The sediment used in the present study was heated to 60°C but was not autoclaved, which may have contributed to the fungal growth observed throughout test development. However, fungal growth was not observed during toxicity tests, which used the same sediment but did not use FFV or the shellfish diet mix. Sterilisation of the sediment was therefore not investigated further, but could be a consideration for future studies to reduce the chances of fungal or bacterial infection.

4.4.1.4 Endpoints

Growth rate was the most sensitive and reliable endpoint we investigated. However, one complication we noted was that the smaller mussels were often killed following exposure to the higher ammonia concentrations, resulting in a higher average length of the larger surviving organisms. This effect was minimised by selecting more suitable sublethal concentration ranges. Dry weight was a problematic endpoint because it was difficult to weigh the very small size mussels accurately (~280 µm starting length, ~21 µg wt), and the sediment and algae adhered to mussel shells, confounding the weights. Wang *et al*. (2011) used dry weight as an additional endpoint to shell growth and survival during 28-d toxicity tests with older juveniles (2-mo-old). The same chronic effect concentration (geometric mean of lowest-observed-effect concentration and NOEC values) of 0.36 mg/L was estimated for growth and dry weight, indicating that either could be used as an endpoint (Wang *et al.* 2011). However, in that study, dry weight data were too variable in their response to be used to calculate an EC10 or EC20 for use in the USEPA (USEPA 2013) ammonia criteria update (Wang *et al*. 2011).

Measuring 14-d survival in the present study was useful as a comparison with other studies, and for determining the magnitude of ammonia concentrations that caused a lethal response. Survival as an endpoint has been compared with growth during 28-d chronic ammonia tests on juvenile mussels by Wang *et al*. (2007c). Chronic values for growth were equal to or lower than survival, indicating that growth rate was the more sensitive endpoint.

4.4.2 Toxicity of ammonia

Velesunio angasi and *Velesunio* sp. showed high chronic sensitivity to ammonia, in comparison to species used in other studies. When sensitivities between mussel species were compared, *Velesunio* sp. from Gulungul Creek were less sensitive than *V. angasi* based on EC50, EC20, and EC10 values. The *V. angasi* from Lake Bennett were slightly more sensitive than *V. angasi* from Sandy Billabong based on EC50 and EC20 values, but of similar sensitivity based on EC10 values. The higher sensitivity of Lake Bennett *V. angasi* was previously observed in acute ammonia toxicity tests with glochidia (Kleinhenz *et al*. 2018). Differences in water quality between Lake Bennett waters and the Magela Creek diluent water were suggested as a possible contributing factor.

The semi-urbanised environment of Lake Bennett may also have affected mussel sensitivity, through increased risk of pollution from domestic wastewater, livestock, or recreational boating activities. However, sensitivity differences between the two different species and three different sites did not vary to the degree found in other studies using multiple mussel species. In a recent multispecies study with temperate juvenile mussels, differences in sensitivity among five species tested with 10 chemicals (including ammonia) were assessed using acute 96-h tests (Wang *et al*. 2017). The EC50 values for all chemicals varied by factors of ≤2 to 12, with ammonia tests varying by a factor of ≤5 (1.5–8.0 mg/L TAN), which was considered low variability (Wang *et al*. 2017). The differences in chronic toxicity estimates from the present study were much lower by comparison, varying at the most by only a factor of 1.6 among all three EC50 values (7.0 – 11.3 mg/L). The small differences between EC50 values from the present study suggest that the sensitivity of the two different local species was relatively comparable, and that the influence of genetic differences on sensitivity was negligible.

The physico-chemistry of the diluent water (Magela Creek water) is likely to be a contributing factor in the high sensitivity of *Velesunio* spp. to ammonia. *Velesunio* spp. glochidia demonstrated high sensitivity to acute ammonia exposures in a previous study using Magela Creek water as the diluent water (Kleinhenz *et al*. 2018), and other studies have

reported increased ammonia sensitivity for other species in similar waters of low ionic strength and low hardness (Ankley *et al.* 1995; Souza-Bastos *et al*. 2017). A recent study by (Mooney *et al*. 2019) assessed chronic ammonia toxicity to six local species from the Alligator Rivers Region. As with the present study, all species were tested in waters of low ionic strength, and were consistently more sensitive to ammonia when compared with international data for species from the same phylogenetic group, and other tropical species. The low ionic strength was hypothesised to increase species' sensitivity to ammonia due to the lack of cations with the capacity to ameliorate toxicity, rather than any physiological differences associated with tropical climatic zones. Further research with *Velesunio* spp. and ammonia under varying ionic conditions may demonstrate the influence of ionic strength on ammonia toxicity in comparison with the effect of pH and temperature.

The comparison of ammonia sensitivity with species used in other chronic studies was dependent on the method used to adjust pH and temperature. Using the USEPA adjustment method (USEPA 2013), *Velesunio* spp. were less sensitive than temperate freshwater mussels and several other temperate taxa, and were highly sensitive compared with other tropical taxa. When the equations of Emerson *et al*. (1975) were used, *Velesunio* spp. were found to be highly sensitive compared with other temperate freshwater mussels and other (non-mussel) temperate and tropical taxa.

Similarly, EC20 data comparisons with chronic ammonia data for the 3 temperate freshwater mussel species (*V. iris*, *L. siliquoidea*, and *Lampsilis fasciola*; Wang *et al*. 2007c, 2011) used in the USEPA ammonia criteria update (USEPA 2013), suggested that *Velesunio* spp. exhibit high sensitivity to ammonia. When EC20 data (Table 4.4) were adjusted to pH 7 and 20°C using the USEPA adjustment method (USEPA 2013), *Velesunio* spp. were found to be slightly less sensitive (EC20s of 5.5–7.6 mg/L TAN; Supplementary Data, Table S4.7) than the highly sensitive *V. iris*, *L. siliquoidea* and *L. fasciola* (adjusted EC20s of 1.4–3.5 mg/L TAN; Wang *et al*. 2007c; 2011), but more sensitive than 16 of the other 18 invertebrate and fish species (EC20s of 3.3–73.7 mg TAN/L; USEPA 2013) used in the update. When adjusted using the equations of Emerson *et al.* (1975), the EC20 values of the present study (Table 4.4) were lower (EC20s of 1.0–1.3 mg/L TAN) than the 3 temperate mussel species (EC20s of 6.4–15.9 mg/L TAN), also indicating high sensitivity. Although comparisons can be made, there were several notable differences in test conditions, for example, the data from Wang *et al*. (2007c, 2011) were derived from 28-d survival tests using 2-mo-old temperate juvenile mussels in flow-through conditions. No chronic data were available for other tropical

freshwater mussel species to allow us to draw further comparisons with the data obtained from the present study.

Based on the findings of Mooney *et al.* (2018), the Emerson *et al.* (1975) equations may more accurately represent values derived in test waters of low ionic strength and low pH, but these authors acknowledged that more data was needed. The USEPA model did not consider ionic strength as a variable affecting ammonia toxicity, and the data used to derive the equations was obtained across a pH range of 6 to 9, with considerable error possible at the lower end of this range (USEPA 1999). Additional chronic ammonia toxicity studies with *Velesunio* spp. using a range of different pH values may reveal the most appropriate data adjustment method for tests conducted in these waters.

The chronic EC10 values for ammonia from the present study (2.78, 2.90, and 3.68 mg/L TAN) can be used with acute toxicity data for *Velesunio* spp. (LC50 = 9.02 mg/L TAN, Kleinhenz *et al.* 2018) to calculate an acute-to-chronic ratio (ACR) of 2.9 for *Velesunio* spp., which is much lower than the ACRs for freshwater mussels provided by the USEPA (2013). The USEPA's ACRs range from 9.03 to 49.5, and were based on acute and chronic data from 4 temperate species, highlighting the variation between chronic and acute responses that can occur between different mussel species (USEPA 2013). This variation demonstrates the inaccuracies in applying a single ACR value across species and that there is no substitute for actual chronic data. Reliance on limited and varying ACR values when deriving site-specific GVs may result in under- or over- protection of species within that environment.

4.5 Conclusion

The chronic toxicity test protocol refined in the present study was shown to be effective for assessing the toxicity of ammonia to tropical freshwater mussels, and showed that *Velesunio* spp. were highly sensitive to ammonia compared with available chronic data for other invertebrate and fish species. *Velesunio* spp. were also similarly sensitive to ammonia compared with limited chronic data for temperate mussel species. The test produced high-quality chronic toxicity estimates for ammonia, which are suitable for deriving water quality guideline values using current best-practice methods in ecotoxicology. The test protocol can be used to assess the toxicity of other contaminants to tropical freshwater mussels, and the data obtained may be incorporated in the revision of national and/or international water quality guideline values.

Acknowledgments

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Supplementary Data

FIGURES

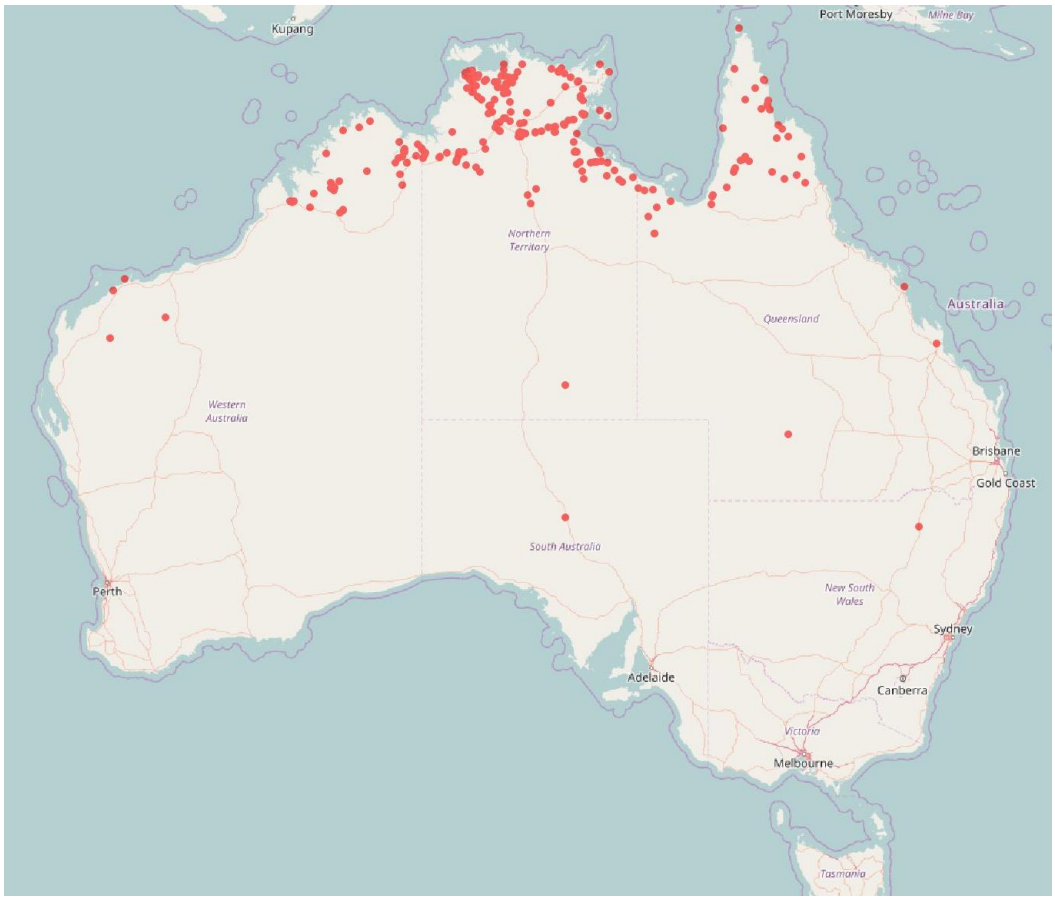

Figure S4.1: Occurrence records (349) for *Velesunio angasi* in Australia as of July 2018. Atlas of living Australia, ala.org.au https://biocache.ala.org.au/occurrences/search?q=lsid:urn:lsid:biodiversity.org.au:afd.taxon:98009b46-32ea-4ae4-83dd-d2c2e9812f51#tab_mapView accessed 14/7/2018

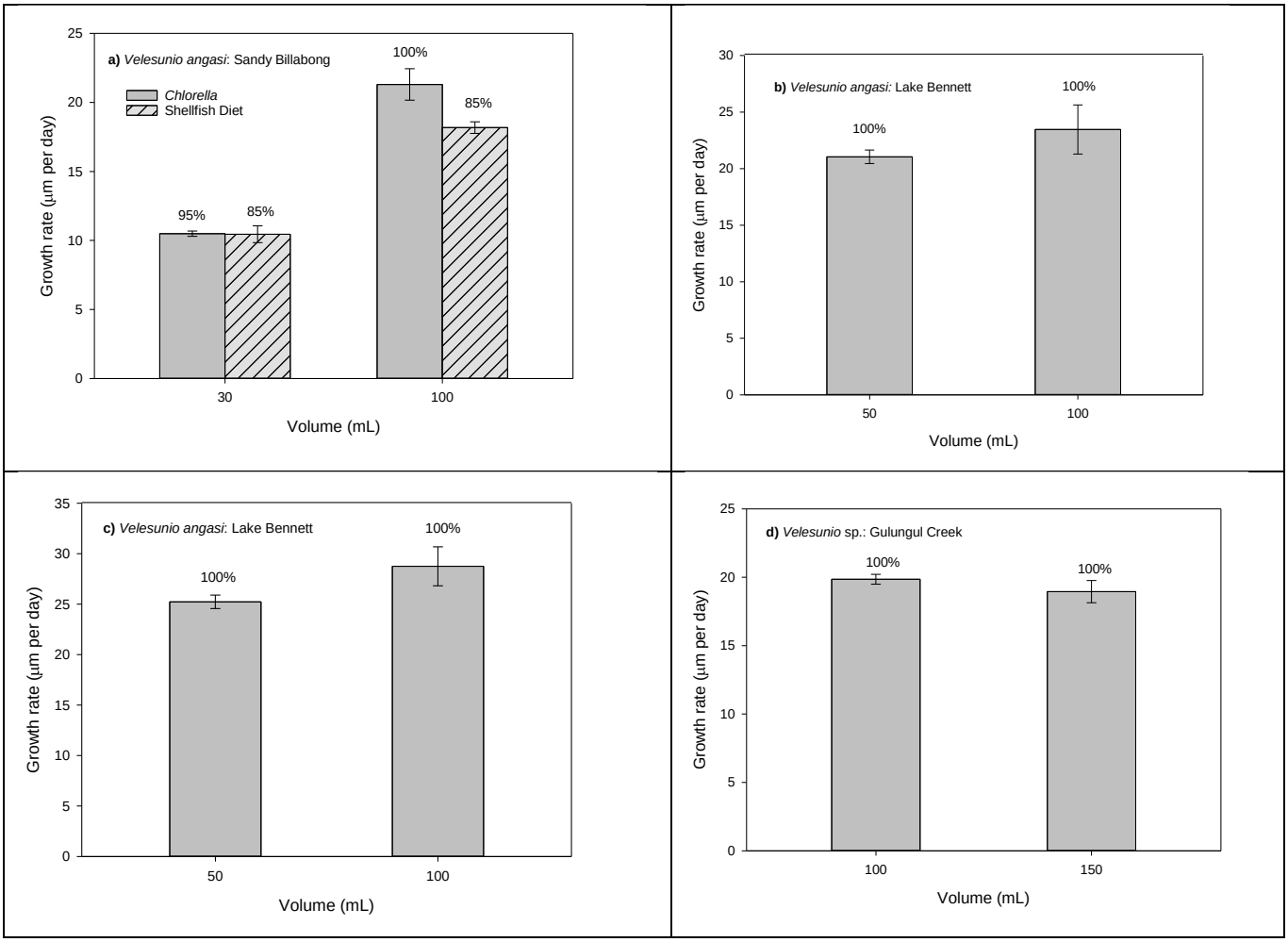

Figure S4.2: Results of four tests comparing growth rates (± SE between two replicates, 10 juveniles per replicate) of both mussel species over 14-d in different test volumes and vessels. Tests were carried out in MCW with algae (*Chlorella* or Shellfish diet ~8 x 10^4 cells/mL). Initial turbidity was either 100 NTU (Tests a and d), or 200 NTU (Tests b and c). Test a: 30 mL in petri dishes vs 100 mL in 175 mL jars, Shellfish diet vs. *Chlorella*, Tests b and c: 50 mL in 75 mL jars vs 100 mL in 175 mL jars with *Chlorella*, Test d: 100 mL in 175 mL jars vs 150 mL in 175 mL jars with *Chlorella*. Percent survival is shown above each column.

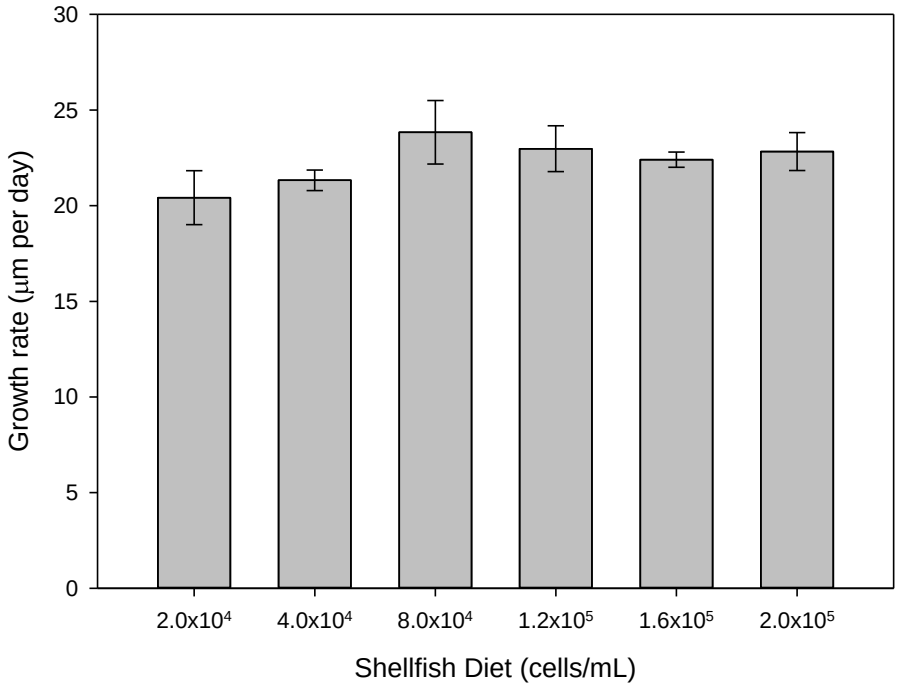

Figure S4.3: Mean growth rate ± standard error (µm/day) of *Velesunio angasi* fed a range of Shellfish Diet volumes. Each data point represents the mean of three replicates ± standard error. Significant difference was seen among treatment groups (P = 0.022) using one-way ANOVA. Pairwise multiple comparisons (Dunn's method) detected significant difference between 20 µL and 80 µL treatments (P = 0.034).

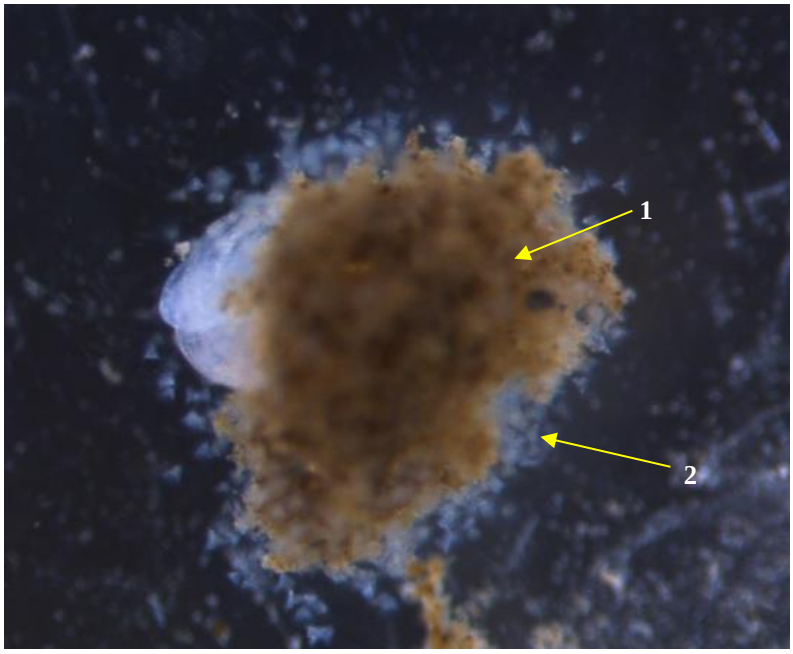

Figure S4.4: Sediment (1) and fungi (2) on a mussel fed shellfish diet and FFV. Image: L. Kleinhenz.

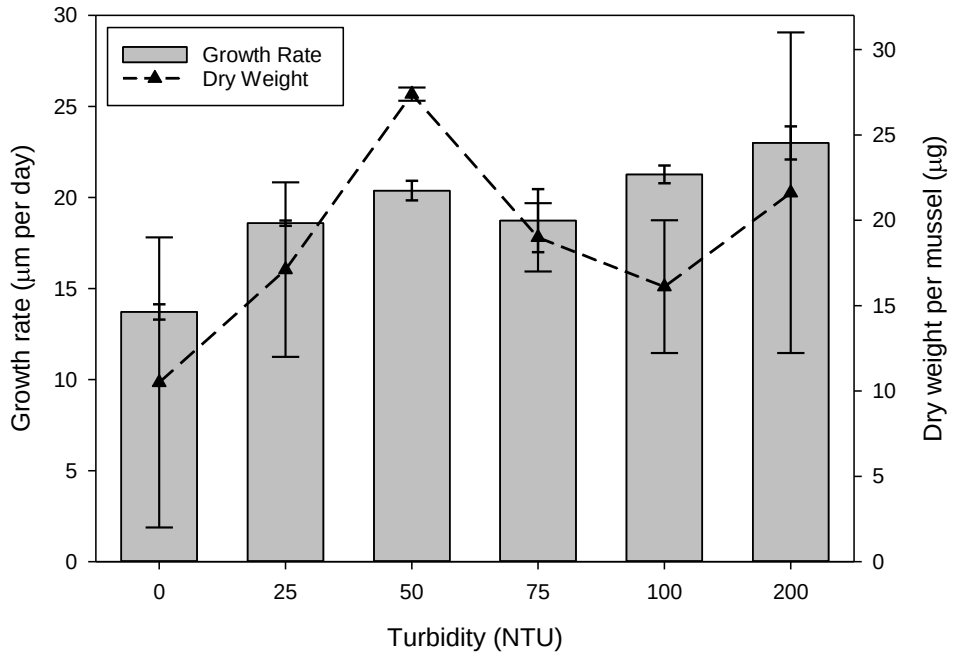

Figure S4.5: Comparison of mean growth rate (left y-axis) and dry weight (right y-axis) as endpoints across a range of turbidities using *Chlorella* sp. as the food source. Data points represent the mean of two replicates ± standard error (*n* = 20 juveniles per replicate).

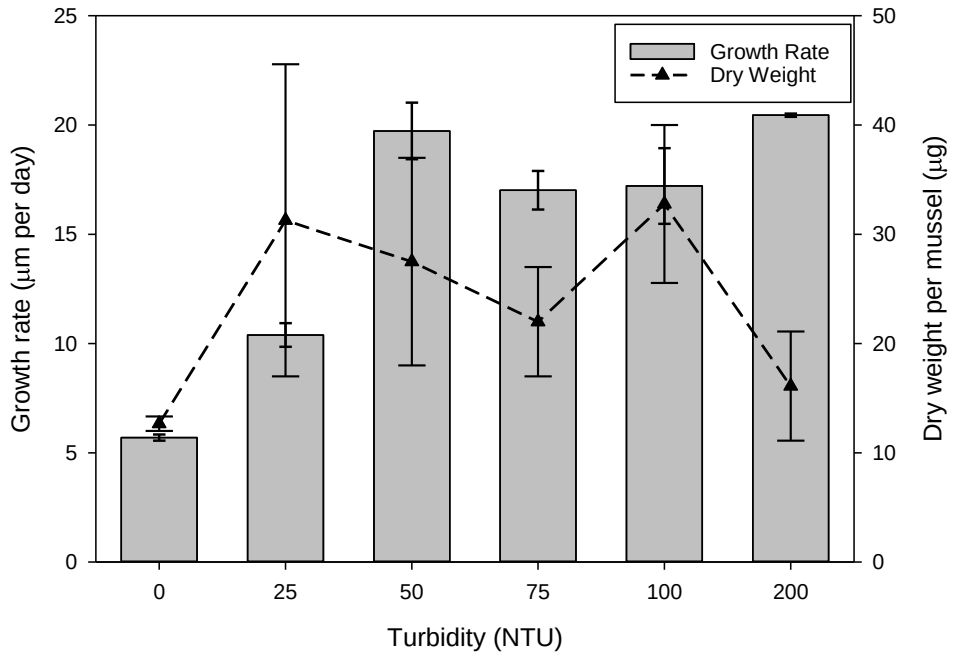

Figure S4.6: Comparison of mean growth rate (left y-axis) and dry weight (right y-axis) as endpoints across a range of turbidities using Shellfish Diet as the food source. Data points represent the mean of two replicates ± standard error (*n* = 20 juveniles per replicate).

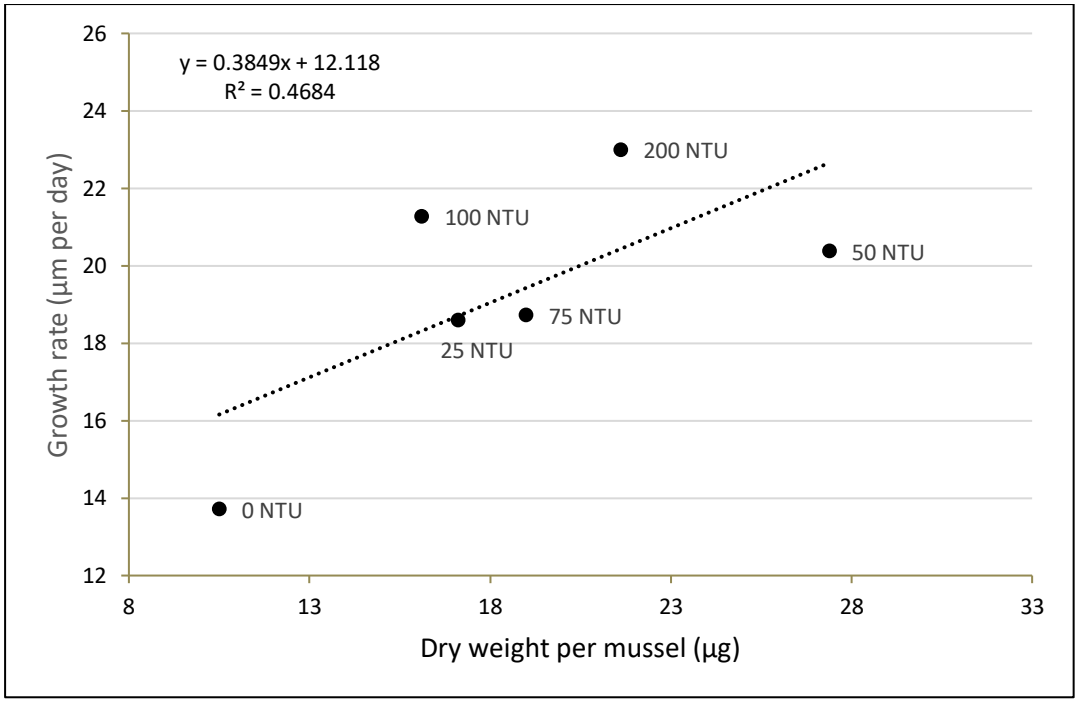

Figure S4.7: Correlation of mean growth rate vs. dry weight as endpoints in a food and turbidity test using *Chlorella* sp. and a range of turbidities. Data points represent the mean of two replicates (*n* = 20 juveniles per replicate).

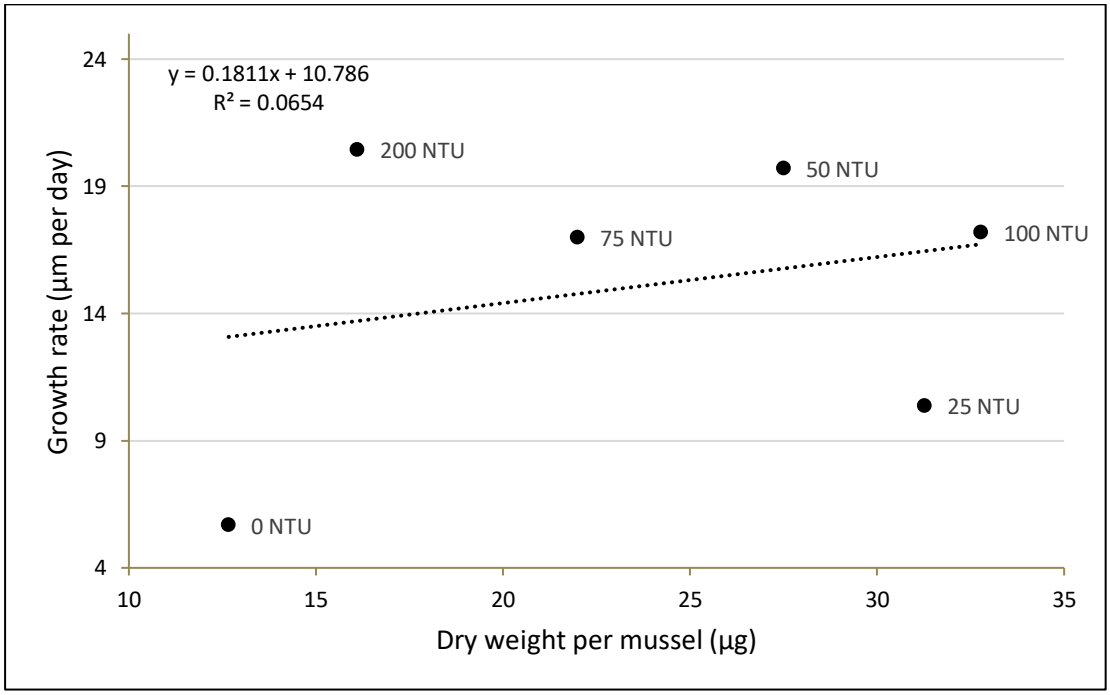

Figure S4.8: Correlation of mean growth rate vs. dry weight as endpoints in a food and turbidity test using Shellfish Diet and a range of turbidities. Data points represent the mean of two replicates (*n* = 20 juveniles per replicate).

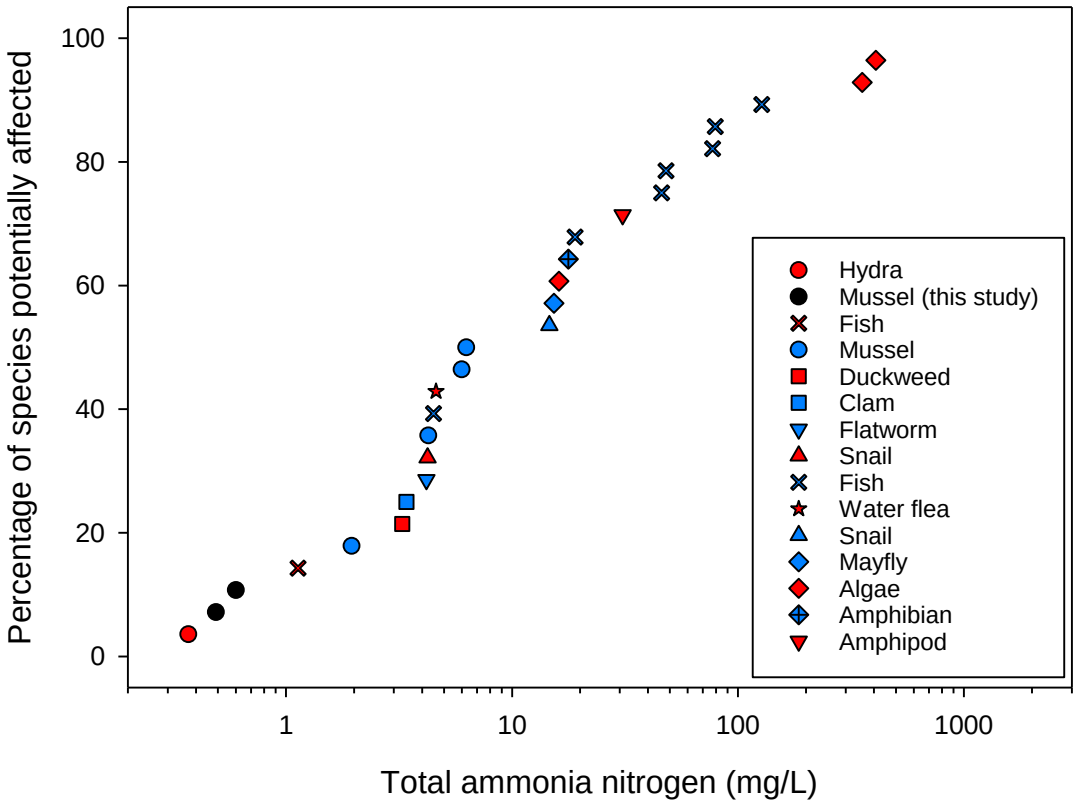

Figure S4.9: Species sensitivity distribution of chronic ammonia toxicity estimates for *Velesunio* spp. from the present study (EC10, black circles), and available chronic EC10/NOEC data for temperate freshwater taxa (blue symbols) and tropical freshwater taxa (red symbols). Data were adjusted to pH 7 and 20°C using the equations of Emerson *et al.* (1975). Original values at test conditions are shown in Supplementary Data, Table S4.6.

TABLES

Table S4.1: Results of growth trials for juvenile *Velesunio* spp. using different foods and algal densities, and exposed to a range of turbidities.

Test #, date	**Mussel species**	**Site**	**Type of food used**	**Algal density (cells/mL)**	**Turbidity** (NTU) [a]	**Growth (**µm per day, mean ± SE	**Survival after 14-d** (%)
1. 26/04/16 & 27/04/16	*Velesunio* sp.	Magela Creek	667 µL *Chlorella*	3.9×10^8	25	13.5 ± 0.4	80
					50	17.8 ± 1.8	85
					100	**22.1 ± 1.2**	85
2. 10/11/15	*V. angasi*	Sandy Billabong	40 µL *Chlorella*	8.0×10^4	0	15.8 ± 0.5	79
					100	**24.3 ± 0.6**	93
					225	19.6 ± 0.9	79
					500	22.1 ± 5.2	100
3. 03/12/16	*V. angasi*	Sandy Billabong	46 µL *Chlorella*	8.3×10^4	0	13.7 ± 0.4	100
					25	18.6 ± 0.1	95
					50	20.4 ± 0.5	95
					75	18.7 ± 1.7	100
					100	21.3 ± 0.5	90
					200	**23.0 ± 0.9**	95
	V. angasi	Sandy Billabong	40 µL shellfish diet (1:10) [b]	7.9×10^4	0	5.7 ± 0.1	95
					25	10.4 ± 0.5	95
					50	19.7 ± 1.3	100
					75	17.0 ± 0.9	100
					100	17.2 ± 1.7	95
					200	**20.5 ± 0.1**	90
4. 18/02/17	*V. angasi*	Lake Bennett	20 µL *Chlorella*	3.2×10^4	50	26.7 ± 1.0	93
					100	28.6 ± 1.9	100
					200	**30.4 ± 1.6**	97
			40 µL *Chlorella*	7.6×10^4	50	27.2 ± 1.7	100
					100	31.6 ± 0.3	90
					200	**32.6 ± 0.9**	90
			80 µL *Chlorella*	1.3×10^5	50	24.5 ± 1.0	93
					100	26.3 ± 0.6	87
					200	**29.1 ± 1.0**	97

[a] Turbidity at commencement of test and at each water change. The turbidity resulting in the greatest growth for each test is highlighted in bold.
[b] Shellfish diet (cell density 2×10^9 cells/mL) was diluted 1:10 first (2×10^8 cells/mL)

Table S4.2: Comparison of the effects of different diets on average juvenile growth (µm per day, ± SE) and survival in treatments at turbidity 100 NTU across 4 different 14-d tests. MC = Magela Creek, SB = Sandy Billabong.

Test #	Test date	Species	Site	Growth with *Chlorella* sp. diet	Survival (%)	Growth with Shellfish diet	Survival (%)	Growth with FFV	Survival (%)
1	21/05/16	*Velesunio* sp.	MC	-	-	31.9 ± 3.6	95	36.6 ± 0.5	85
1	21/05/16	*Velesunio* sp.	MC	-	-	33.5 ± 0.9	100	39.2 ± 0.3	75
2	26/04/16	*Velesunio* sp.	MC	22.1 ± 1.2	85	-	-	29.7 ± 0.3	70
3	03/12/17	*V. angasi*	SB	21.3 ± 0.5	90	17.2 ± 1.7	95	-	-
4	05/12/17	*V. angasi*	SB	21.3 ± 1.1	100	18.2 ± 0.4	85	-	-

Table S4.3: Mean growth rate and survival of juvenile *Velesunio angasi* using different water change regimes (*n* = 10 juveniles per replicate).

Test #	Test date	Species	Site	Treatment	Growth rate (µm/day, mean ± SE)	Survival (%)	pH	EC	DO
1	26/04/16	*Velesunio* sp.	MC	Water changed every 2 days	22.1 ± 1.2 [a]	85	6.4 (± 0.3)	13.5 (± 0.52)	98.9 (± 5.1)
2	28/04/16	*Velesunio* sp.	MC	A – Water changed on days 4, 8, & 12	15.8 [b]	70	6.2 (± 0.2)	13.0 (± 0.76)	98.9 (± 2.4)
2	28/04/16	*Velesunio* sp.	MC	B – Water changed on day 7 only	13.2 [b]	70	6.2 (± 0.2)	13.0 (± 0.82)	99.3 (± 2.9)

[a] Two replicates.
[b] Only one replicate was used due to the low numbers of juveniles available.

Table S4.4: Growth, survival, and water quality of juveniles exposed to HEPES and MES buffers.

Test date	Species	Site	Treatment	Av. Growth per day (µm)	Survival (%)	pH (av.)	EC	DO
21/05/16	*Velesunio* sp.	MC	HEPES	31.9 ± 3.56	95	6.1 ± 0.1	25.9 ± 2.5	94.2 ± 4.7
			MES	33.5 ± 0.87	100	6.0 ± 0.1	59.4 ± 1.6	94.5 ± 4.5

Table S4.5: Nominal and measured ammonia concentrations (mg/L TAN) for all ammonia tests. GC = Gulungul Creek, LB = Lake Bennett, SB = Sandy Billabong.

Test	Treatment	Nominal	Measured (start of test)	Measured (end of test)	Measured (average)	Measured % change
1571M	A - Control	0	0.05	0.01	0.03	
GC	B	2	1.92	1.77	1.84	-7.72
	C	4	3.44	3.89	3.66	13.02
	D	8	7.78	8.13	7.95	4.53
	E	12	9.55	11.47	10.51	20.10
	F	16	15.39	15.87	15.63	3.12
	G	24	23.28	24.67	23.98	5.98
	H	32	31.10	31.10	31.10	0.00
1574M	A - Control	0	0.00	0.00	0.00	
LB	B	2.5	2.61	2.07	2.34	-20.73
	C	5	5.01	4.36	4.69	-12.97
	D	7.5	7.74	6.87	7.31	-11.24
	E	10	10.06	8.98	9.52	-10.74
	F	12.5	13.80	11.28	12.54	-18.30
	G	15	15.96	14.10	15.03	-11.65
	H	17.5	18.17	16.66	17.41	-8.29
	I	20	20.28	18.16	19.22	-10.45
1585M	B - Control	0	0.31	-0.04	0.14	
GC	C	1.5	2.09	1.88	1.98	-10.07
	D	3	4.36	3.93	4.15	-9.90
	E	4	5.91	5.59	5.75	-5.41
	F	6	8.93	8.59	8.76	-3.76
	G	12.5	18.40	18.90	18.65	2.72
	H	17	25.50	25.77	25.64	1.07
1587M	B - Control	0	0.08	0.02	0.05	
LB	C	1	0.85	0.78	0.82	-8.43
	D	3	3.07	2.90	2.99	-5.47
	E	6	6.16	6.14	6.15	-0.19
	F	8	7.87	8.00	7.94	1.63
	G	10	9.92	10.28	10.10	3.63
	H	15	14.52	15.54	15.03	7.02

Table S4.5: (Continued)…

1597M	B - Control	0	0.08	0.01	0.04	
SB	C	1	1.06	0.84	0.95	-21.13
	D	2	2.03	1.86	1.94	-8.66
	E	4	4.00	3.76	3.88	-6.00
	F	6	6.23	5.75	5.99	-7.71
	G	8	8.08	8.02	8.05	-0.79
	H	10	10.30	10.34	10.32	0.39
	I	12	11.98	11.76	11.87	-1.80
	J	16	16.58	16.35	16.46	-1.35
1605M	A - Control	0	0.03	0.02	0.02	
SB	B	1.5	1.50	1.36	1.43	-8.95
	C	3	3.10	2.91	3.00	-6.19
	D	5	4.93	4.75	4.84	-3.65
	E	7	7.07	6.94	7.01	-1.84
	F	9	9.28	8.68	8.98	-6.47
	G	14	13.98	13.72	13.85	-1.86
	H	18	18.83	18.98	18.90	0.80
	I	20	20.70	20.45	20.58	-1.21

Table S4.6: Chronic ammonia toxicity data comparisons with tropical and temperate species showing the original ammonia toxicity value (EC_{10} or NOEC) reported, the NH3 concentration, and adjustments made to pH 7 at 20°C using two different methods.

Species name	**Test duration / Endpoint**	**NH3** (µg/L) At test conditions	**EC10** (mg/L TAN) [a] 24-h tests at test conditions (pH, °C)	**EC10** (mg/L TAN) [b] At pH 7, 20°C (Emerson *et al*. 1975)	**EC10** (mg/L TAN) [c] At pH 7, 20°C (USEPA method)	**Reference**
			Tropical species			
Algae *Chlorella vulgaris*	72 h EC10 (Growth rate)	1950	712.97 (pH 6.6, 25°C)	407.36	885.88	Wang and Leung (2015)
Algae *Pseudokirchneriella subcapotatum*	72 h EC10 (Growth rate)	1700	621.56 (pH 6.6, 25°C)	355.13	772.30	Wang and Leung (2015)
Algae *Chlorella sp.*	72 h EC10 (Growth rate)	77.17	66.03 (pH 6.1, 29.3°C)	16.12	102.8	Mooney *et al*. (2019)
Duckweed *Lemna aequinoctialis*	96 h EC10 (surface growth)	15.64	22.40 (pH 5.9, 28.5°C)	3.27	32.82	Mooney *et al*. (2019)
Green hydra *Hydra viridissima*	96 h EC10 (population growth)	1.38	1.70 (pH 6, 27.4°C)	0.29	2.33	Mooney *et al*. (2018)
Green hydra *Hydra viridissima*	96 h EC10 (population growth)	2.27	1.82 (pH 6.2, 26.9°C)	0.47	2.44	Mooney *et al*. (2019)
Amphipod *Hyalella azteca*	10 w NOEC (Reproduction)	148	2.08 (pH 8.04, 25.0°C)	30.92	7.40	Borgmann (1994)
Water flea *Moinodaphnia macleayi*	3 brood (Reproduction)	22.07	26.92 (pH 6.0, 27.5°C)	4.61	37.13	Mooney *et al*. (2019)
Snail *Amerianna cumingi*	14 d EC10 (Reproduction)	20.23	16.73 (pH 6.1, 29.8°C)	4.23	26.90	Mooney *et al*. (2019)
Mussel *Velesunio* sp**.**	14-d EC10 (Growth)	2.89	3.68 (pH 5.99, 27.2°C)	0.60	4.98	This study
Mussel *Velesunio angasi*	14-d EC10 (Growth)	2.44	2.90 (pH 6.02, 27.2°C)	0.51	3.93	This study
Mussel *Velesunio angasi*	14-d EC10 (Growth)	2.34	2.78 (pH 6.02, 27.2°C)	0.49	3.76	This study
Fish *Mogurnda mogurnda*	7 d (Growth)	5.41	5.39 (pH 6.1, 27.1°C)	1.13	4.61	Mooney *et al*. (2019)

Temperate species						
Mussel *Villosa iris*	28 d NOEC (Growth)	<28.67	<0.40 (pH 8.2, 20.0°C)	<5.99	<0.74	Wang *et al.* (2007c)
Mussel *Lampsilis fasciola*	28 d NOEC (Growth)	<9.32	<0.13 (pH 8.2, 20.0°C)	<1.95	<0.43	Wang *et al.* (2007c)
Mussel *Lampsilis siliquoidea*	28-d NOEC (Growth)	20.39	0.25 (pH 8.26, 20°C)	4.26	0.91	Wang *et al.* (2011)
Mussel *Musculium transversum*	42 d NOEC (Survival)	30	0.89 (pH 7.8, 21.8°C)	6.27	1.86	Sparks and Sandusky (1981)
Clam *Sphaerium novaezelandiae*	60 d NOEC (Survival)	16.31	0.97 (pH 7.55, 20.0°C)	3.41	1.37	Hickey and Martin (1999)
Flatworm *Polycelis feline*	30 d NOEC (Survival)	20	0.50 (pH 8.1, 15.0°C)	4.18	1.01	Alonso and Camargo (2011)
Snail *Potamopyrgus antipodarum*	40 d NOEC (Immobility)	70	1.74 (pH 8.1, 15.0°C)	14.62	3.54	Alonso and Camargo (2009)
Mayfly *Deleatidium sp.*	29 d NOEC (Survival)	73.36	0.949 (pH 8.37, 15.81°C)	15.32	3.16	Hickey and Martin (1999)
Fish *Micropterus dolomieue*	32 d NOEC (Biomass)	21.5	9.53 (pH 6.6, 22.3°C)	4.49	8.58	Broderius *et al.* (1985)
Fish *Lepomis cyanellus*	44 d NOEC (Biomass)	220	5.16 (pH 7.9, 22.0°C)	45.96	10.91	McCormick *et al.* (1984)
Fish *Deltistes luxatus*	30 d NOEC (Survival)	370	0.51 (pH 9.5, 22.3°C)	77.29	11.20	Meyer and Hansen (2002)
Fish *Esox lucius*	52 d EC20 (Biomass)	91	10.89 (pH 7.62, 8.6°C)	19.01	16.52	Harrahy *et al.* (2004)
Fish *Xyrauchen texanus*	28 d LC20 (Survival)	380	4.86 (pH 8.24, 20.0°C)	79.38	17.09	Fairchild *et al.* (2005)
Fish *Notropis topeka*	30 d NOEC (Growth)	230	7.62 (pH 8.07, 12.0°C)	48.05	20.53	Aldelman *et al.* (2009)
Fish *Ptychocheilus lucius*	28 d LC20 (Survival)	610	7.81 (pH 8.24, 20.0°C)	127.43	27.44	Fairchild *et al.* (2005)
Amphibian *Pseudacris regilla*	10 d NOEC (Biomass)	85	30.59 (pH 6.7, 22.0°C)	17.76	28.06	Schuytema and Nebeker (1999)

[a] Data reported in NH3 were adjusted to TAN using the speciation equations of Emerson *et al*. (1975)

[b] Data were adjusted to pH 7 and 20°C using the speciation equations of Emerson *et al*. (1975).

[c] Data were adjusted to pH 7 and 20°C using the speciation equations of Emerson *et al*. (1975), and USEPA (2013).

Table S4.7: Summary of the ammonia toxicity estimates for tests with *Velesunio* sp. (Gulungul Creek), and *Velesunio angasi* (Lake Bennett and Sandy Billabong), adjusted to pH 7 and 20°C using the method of USEPA (2013).

		Total Ammonia Nitrogen (mg L-1)		
Species	Site	EC10	EC20	EC50
Velesunio sp.	Gulungul Creek	4.98	7.61	15.24
V. angasi	Lake Bennett	3.93	5.50	9.47
V. angasi	Sandy Billabong	3.76	5.93	12.42

CHAPTER 5: Acute and chronic toxicity of magnesium to the early life stages of two tropical freshwater mussel species.

Abstract

Magnesium (Mg) is a common contaminant in mine water discharges. Although Mg is an essential element in biological processes, increased concentrations from anthropogenic sources can stress aquatic ecosystems. Additionally, studies evaluating the effects of Mg on north Australian freshwater species have indicated that in very soft waters there is a high risk to some species. Freshwater mussels are an ecologically and culturally important taxon in many freshwater environments, but knowledge of their sensitivity to Mg is limited. In the present study, the acute and chronic sensitivity of two freshwater mussel species, *Velesunio angasi* and an undescribed *Velesunio* species, to Mg was assessed (using $MgSO_4$) on their early life stages, larval glochidia and post-parasitic juveniles. Acute 24-h exposures with glochidia generated a mean median lethal (LC50) toxicity estimate of 284 mg/L for the five tests with *V. angasi*¸ and a mean LC50 of 300 mg/L for the three tests with *Velesunio* sp. Mean chronic 14-d toxicity estimates resulting in 50% (EC50) and 10% (EC10) growth rate reductions for juveniles were 241 and 88 mg/L respectively for the three tests with *V. angasi* juveniles, and 232 and 87 mg/L respectively for the three tests with *Velesunio* sp. juveniles.

The results represent the first acute and chronic Mg toxicity data for tropical freshwater mussels, and indicated that *V. angasi* and *Velesunio* sp. exhibited similar sensitivity and were moderately sensitive to Mg when compared to other tropical species. These results are a valuable contribution to the small existing dataset for Mg toxicity to tropical freshwater species, which can be used to inform water management in areas where Mg is a contaminant of concern, and ensure the protection of these taxa.

Keywords: Guideline values, salinity, ionic strength, mining, aquatic invertebrates, bivalves

5.1 Introduction

Magnesium (Mg) is a common mining-derived contaminant in freshwater ecosystems, originating through the leaching of minerals from waste rock (Sinclair *et al.* 2013; Griffith *et al*. 2012), and controlled releases of mining wastewater (van Dam *et al.* 2010). Other anthropogenic sources of Mg include fertilisers, liming, and alloys for many industrial products such as car and aircraft parts, titanium, aluminium, and high-grade steel (USGS 2018). In living organisms, Mg is essential for numerous metabolic processes such as oxidative phosphorylation, glycolysis, protein synthesis, electrolyte transport across cell membranes, and plant photosynthesis (Wolf and Cittadini 2003, Maguire and Cowan 2002, Pasternak *et al*. 2010).

Along with other ions, Mg is a major contributor to increasing concentrations of dissolved inorganic ions (salinisation) in water or soil (Williams 1987; Cañedo-Argüelles *et al*. 2013). Although the salinisation of river systems occurs naturally, anthropogenic salinisation is an emerging global issue (Kefford *et al*. 2016). For example, high salinity coal-mining wastewaters entering freshwater streams in the USA and Australia have been shown to increase the downstream concentrations of major ions, such as sulfate (SO_4^{2-}), bicarbonate (HCO_3^-), calcium (Ca^{2+}), and magnesium (Mg^{2+}), which may cause adverse impacts on freshwater biota and ecosystems (Griffith *et al.* 2012; Cormier *et al*. 2013; Dunlop *et al*. 2015). The main toxic effect of elevated ion concentrations on freshwater invertebrates is cellular damage caused by osmo-regulatory stress (Kefford *et al.* 2016) occurring through direct exposure of gills and other respiratory surfaces to dissolved ions in the water (USEPA 2011). However, direct toxicity of major ions through non-osmoregulatory mechanisms can also occur (see van Dam *et al*. 2010). Tolerance to increased salinity and individual ions can be very species-specific (Soucek and Kennedy 2005; Griffith 2017), and it is unknown if individual ions or a combination of ions are involved in determining the upper salinity limit of species (Cañedo-Argüelles *et al*. 2013).

In contrast to waters of moderate to high hardness (Mg^{2+} and Ca^{2+} ions), which have been shown to reduce the toxicity of metal contaminants such as copper (Gillis *et al.* 2008) and uranium (Riethmuller *et al*. 2001, Markich 2013), waters with low ionic strength can increase the uptake and toxicity of metal and major ion contaminants in aquatic species, due

to a lack of ameliorative ions (Oliveira-Filho *et al.* 2014). The toxicity of small concentrations of Mg in these low ionic strength waters may be largely due to its properties as a Ca channel antagonist in aquatic organisms (van Dam *et al.* 2010).

In tropical northern Australia, mine seepage waters rich in $MgSO_4$ have been associated with impairment of aquatic life in water bodies close to mining operations (Humphrey and Chandler 2018; Supervising Scientist 2018a). Laboratory toxicity studies using $MgSO_4$ found that, while SO_4 contributed slightly to toxicity, the Mg ion was far more toxic (at least 17–50 times based on mass) to tropical freshwater invertebrates resident in the surrounding low ionic content surface waters (van Dam *et al*. 2010).

Varying ratios of Mg^{2+} and Ca^{2+} ions in exposure water can modify the toxicity of some contaminants to some freshwater species, with ameliorative effects attributed to increased Ca^{2+} (van Dam *et al*. 2010; Luo *et al*. 2016; Mount *et al*. 2016; Soucek *et al.* 2018). However, the extent of Ca amelioration of Mg toxicity has been shown to be organism dependent (Humphrey and Chandler 2018; Trenfield *et al*. 2018; Supervising Scientist 2018a), and some species may not be protected by Ca amelioration in these low ionic strength water bodies.

A site-specific water quality guideline value (GV) of 2.5 mg/L (99% species protection level) has been derived for Mg (at a Mg:Ca ratio of $\leq$ 9:1) based on the responses of six aquatic species local to the Alligator Rivers Region of north Australia. This ratio was found to be the highest ratio that still provided protection for the species tested (van Dam et al. 2010).

Freshwater mussels are one of the most increasingly imperilled aquatic groups worldwide, and environmental pollution has been implicated in many species' declines (Ferreira-Rodriguez *et al*. 2019). Their unique lifecycle involves an obligate parasitic stage, where the glochidia (larvae) attach to a suitable host fish to complete development into juveniles (Kat 1984). These early life stages are typically vulnerable to contaminants in the water-column and sediment. Studies using standardised toxicity testing methods (ASTM 2006) have reported high sensitivity of temperate freshwater mussels in their early life stages to other contaminants, particularly copper and ammonia (Wang *et al.* 2007a; Wang *et al*. 2007c; Gillis *et al*. 2008; Clearwater *et al*. 2014; Markich 2017). However, chronic sub-lethal toxicity data used for developing GVs for contaminants are generally under-represented for

freshwater mussels and, in particular, acute and chronic toxicity data are lacking for tropical species (Kwok *et al*. 2007).

In Australia and New Zealand, a key approach for protecting aquatic ecosystems from contaminants is through the development and application of water quality GVs, which aim to ensure that harmful contaminant concentrations are not exceeded. Environmental variables that influence natural water quality mean that generic water quality GVs cannot always provide site-specific protection (van Dam *et al.* 2017).

Site-specific GVs based on local species' toxicity data are preferred over default (generic) GVs, but are often based on small sample sizes, which increases the uncertainty of such locally derived water quality criteria. Thus, any ability to increase the number of local species that can be used for deriving a site-specific GV will improve the confidence in the GV (ANZG 2018).

Currently no acute or chronic Mg toxicity data are available for tropical freshwater mussels. Recent studies have assessed the acute and chronic toxicity of ammonia to two tropical freshwater mussel species, *Velesunio angasi* and *Velesunio* sp., (Kleinhenz *et al*. 2018, Kleinhenz *et al*. 2019a, acute and chronic respectively), the protocols of which are suitable for assessing the toxicity of other contaminants, such as Mg, to tropical mussels.

The aim of this study was to use recently optimised toxicity test protocols of Kleinhenz *et al*. (2018, 2019a) to assess the acute and chronic toxicity of Mg to the early life stages of *Velesunio* spp., in waters of low ionic strength. The results will address gaps in the dataset for tropical freshwater species and major ion toxicity in low ionic strength waters. This will improve the confidence that aquatic ecosystems, including the ecologically and culturally important mussels, will be appropriately protected.

5.2 Materials and methods

5.2.1 Study area

The Alligator Rivers Region (ARR), an area of approximately 28,000 km^2 located in tropical northern Australia, was the main area from which mussels were collected for this study (Figure 5.1). Included within the ARR are areas of high conservation value, internationally recognised for their cultural and ecological significance (Sinclair *et al.* 2013). Legislation and continuing research ensure that high levels of environmental protection from uranium mining activities are in place (van Dam *et al*. 2002). Contaminants of potential concern such as magnesium sulfate may enter the adjacent Magela Creek system during controlled releases of minimally contaminated mine water, and through groundwater discharge during the wet season (van Dam *et al*. 2004; van Dam *et al*. 2010). The natural surface waters of the region are typically slightly acidic pH (~5.5–6.5), with low electrical conductivity (~5–20 µS/cm), hardness (~3–6 mg/L as $CaCO_3$), and alkalinity (5–10 mg/L as $CaCO_3$).

5.2.2 Test organisms

The mussel species used in the present study belong to the genus *Velesunio* (Hyriidae family) and are found in waterways throughout the wet-dry tropics of northern Australia. The lentic *Velesunio angasi* is found in permanent waterbodies, including billabongs and lakes, and may reproduce year-round if conditions are favourable (Humphrey and Simpson 1985). Humphrey and Simpson (1985) also studied mussels inhabiting creeks and streams that cease to flow during the dry season, undergoing a period of dormancy by burrowing into the sandy banks of the dry creek beds before flows cease in the early dry season. While they assumed this mussel form was a phenotypic variant of *Velesunio angasi*, recent molecular analysis (Environmental Research Institute of the Supervising Scientist, Australian Department of the Environment and Energy, Canberra, ACT, Australia and Griffith University, Brisbane, Qld, Australia, unpublished data) has determined that this lotic form is a separate species, henceforth referred to as *Velesunio* sp.

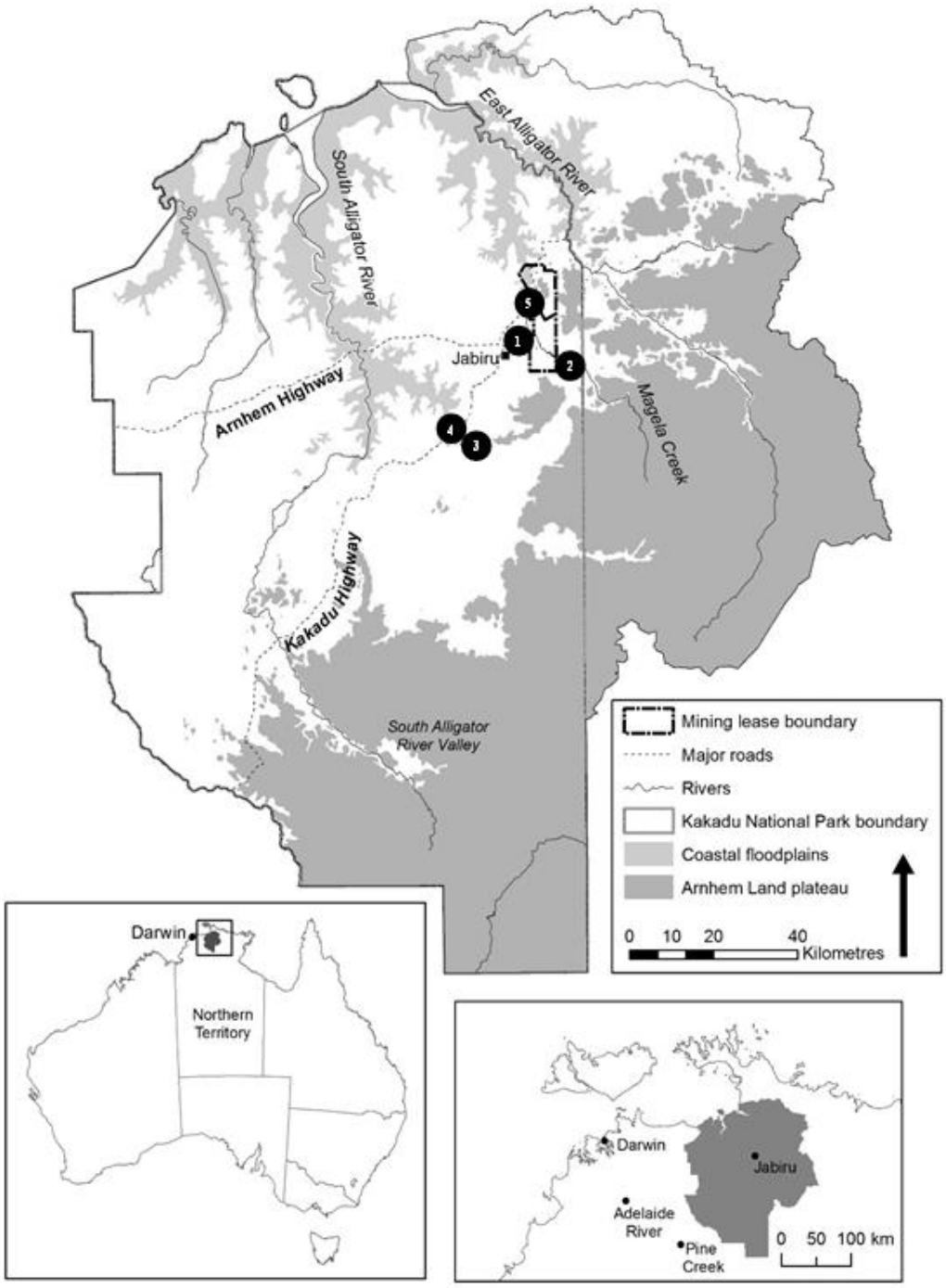

Figure 5.1: The Alligator Rivers Region of the Northern Territory, Australia, showing mussel collection sites. 1: Gulungul Creek, 2: Magela Creek, 3: Sandy Billabong, 4: Nourlangie Creek, 5: Mudginberri Billabong (map used with permission, Supervising Scientist 2015).

Mussels were collected between October 2017 and June 2018 for all tests. *Velesunio angasi* was collected from three sampling sites within the Alligator Rivers Region (Figure 5.1): Sandy Billabong (latitude 12° 54' 4''S, longitude 132° 46' 38''E), Nourlangie Creek (latitude 12°51'22''S, longitude 132°46'38''E), and Mudginberri Billabong (latitude 12° 35' 33''S, longitude 132° 52' 33''E), and one site outside of the ARR: Lake Bennett (latitude 12° 57'33''S, longitude 131° 9' 53''E), a semi-urbanised man-made lake situated 80 km south of Darwin. *Velesunio* sp. was collected from two sampling sites within the Alligator Rivers Region (Figure 5.1): Gulungul Creek (latitude 12°39'21''S, longitude 132°52'42''E), and Magela Creek (latitude 12° 40' 58.93''S, longitude 132° 56' 23.30''E). Mussels were transported live to the laboratory at ambient temperature (28–34°C) in 20 L aerated plastic drums containing ~15 L of water from the collection site, within 4 h of collection. In the laboratory, glochidia were isolated from adult female mussels and checked for viability according to the method detailed in Kleinhenz *et al*. (2018). Viable mature glochidia were used for acute toxicity testing or host-fish exposure within 24 h of isolation from adult female mussels. To obtain juvenile mussels for chronic tests, mature glochidia were metamorphosed into juvenile mussels in the laboratory using the northern trout gudgeon, *Mogurnda mogurnda*, as a host fish. The methods for host-fish exposure, and collection and selection of juveniles for use in toxicity tests are detailed in Kleinhenz *et al*. (2019a). Newly transformed juveniles were used for chronic toxicity tests within 24 h of excystment from host fish.

5.2.3 Test diluent

The test diluent, natural Magela Creek water, was collected monthly from Bowerbird Billabong (latitude 12°46'18''S, longitude 133°02'23''E), a permanent billabong in the ARR that is upstream of any significant human activity. The water was collected in 20 L acid-washed polyethylene containers and transported to the laboratory at ambient temperature (28–32°C) within 4 h of collection. The water was filtered on arrival (Minisart RC 25, 0.45 µm filter, Sartorius Stedim), then stored at 4°C for use in toxicity tests within one month following collection. The physico-chemical properties of the Magela Creek water measured during the study are shown in Table 5.1.

Table 5.1: Average physico-chemical composition of 0.45 µm filtered Magela Creek water (MCW) used in definitive $MgSO_4$ acute tests (n = 8), and chronic tests (n = 6). Values represent the mean measurements ± standard deviation of control samples from each test.

Physicochemical variable		**Acute tests**	**Chronic tests**
pH		6.05 ± 0.03	6.06 ± 0.04
Conductivity (µS/cm)		15.6 ± 1.7	16.2 ± 1.9
Dissolved oxygen (%)		103.6 ± 6.6	110.4 ± 5.4
Alkalinity (mg/L as $CaCO_3$)		<1.0	1.4 ± 0.9
Hardness (mg/L as $CaCO_3$)		3.0 ± 1.0	2.9 ± 0.7
DOC (mg/L)		2.8 ± 0.9	2.5 ± 0.8
Analyte	**Detection limit**		
Al (µg/L)	0.1	26.5 ± 27.6	18.3 ± 7.8
Cd (µg/L)	0.02	<0.02	<0.02
Co (µg/L)	0.01	0.06 ± 0.01	0.11 ± 0.04
Cr (µg/L)	0.1	0.14 ± 0.05	0.12 ± 0.04
Cu (µg/L)	0.01	0.30 ± 0.22	0.15 ± 0.04
Fe (µg/L)	1	60.4 ± 6.4	85.0 ± 37.9
Mn (µg/L)	0.01	2.03 ± 0.72	6.20 ± 1.77
Ni (µg/L)	0.01	0.81 ± 0.80	0.19 ± 0.07
Pb (µg/L)	0.01	0.11 ± 0.09	0.04 ± 0.01
Se (µg/L)	0.2	<0.2	<0.2
U (µg/L)	0.001	0.073 ± 0.065	0.014 ± 0.008
Zn (µg/L)	0.1	3.45 ± 2.81	0.47 ± 0.16
Ca (mg/L)	0.1	0.15 ± 0.05	0.13 ± 0.05
Mg (mg/L)	0.1	0.63 ± 0.23	0.63 ± 0.15
Na (mg/ L)	0.1	1.81 ± 0.29	1.80 ± 0.14
SO_4 (mg/L)	0.5	93.8 ± 11.2	93.0 ± 2.4

5.2.4 Preparation of test solutions

All equipment used for toxicity testing was made from chemically inert materials and prepared according to the procedures detailed in Kleinhenz *et al*. (2018). All reagents used were analytical grade. Stock solutions of 40 g/L Mg for toxicity tests were prepared by dissolving $MgSO_4.7H_2O$ (Ajax Finechem) in high-purity water (18 MΩ/cm, Milli-Q, Millipore). Calcium was added to reach a nominal Mg:Ca (mass) ratio of 9:1, which was chosen based on the findings of van Dam *et al*. (2010), which showed that when the Mg:Ca ratio was higher (Mg increased), the toxicity of Mg increased significantly for some species without the protective properties of calcium. Using a Mg:Ca ratio of 9:1 was considered to be environmentally relevant in the extremely soft waters surrounding the mine, and the current operational limit of 3.0 mg/L Mg for the Ranger mine also requires that the Mg:Ca of 9.1 is not exceeded (Supervising Scientist 2018a).

Calcium stock was prepared by adding $CaSO_4.2H_2O$ (Chem-Supply) to Magela Creek water at a concentration of 1.1 g/L, stirring and leaving overnight to dissolve. Test solutions were prepared by diluting stock solutions with Magela Creek water (nominal $MgSO_4$ concentration ranges 0 – 500 mg/L), and adding 1 mM HEPES buffer (N-2-hydroxy-ethylpiperazine-N0-2-ethanesulfonic acid, Sigma-Aldrich). The pH of the test solutions was adjusted using 5% sodium hydroxide (NaOH) or 5% sulfuric acid (H_2SO_4) if necessary, to ensure the starting pH was close to the environmentally relevant pH of 6.0 ± 0.1.

5.2.5 Toxicity tests

Eight acute toxicity tests were undertaken using mussel glochidia and the 24-h toxicity test described by Kleinhenz *et al*. (2018), and six chronic toxicity tests were undertaken using juvenile mussels and the 14-d toxicity test described by Kleinhenz *et al*. (2019a).

For the acute tests, there were three replicates per treatment, with each replicate containing ten glochidia. The survival endpoint was measured at the end of the tests by observing valve closure after adding 2 to 3 drops of a concentrated (240 g/L NaCl) salt solution to each replicate (ASTM 2006). Glochidia that were already closed before the addition of salt, or those that remained open after the addition of salt, were counted as functionally dead.

Chronic tests consisted of two replicates per treatment, with each replicate containing ten newly-excysted juvenile mussels. Mussels were fed every 48 h with ~8.0 x 10^4 cells/mL of the laboratory cultured green alga, *Chlorella* sp., and sediment that had been collected from Sandy Billabong was added in the form of a fine (< 63 µm) silt to provide optimal conditions for feeding and growth (Hudson and Isom 1984). Growth rate was measured by photographing mussels on days 0, 8 and 14 using a microscope fitted with a camera (Leica MC170HD camera, Leica M205C microscope at 8.0 x magnification), and using the Leica image analysis software to measure the maximum shell length of each mussel. For starting (day 0) measurements, the average length was calculated for a separate sub-sample of 30 juveniles to avoid potential damage to mussels used in testing. Mussels were euthanised with 70% ethanol at the end of each test and excess sediment removed to aid in accurate and efficient measurements. Further details of the acute and chronic test protocols used in this study are provided in Table 5.2.

5.2.6 Physico-chemical analyses

Unfiltered and 0.45 µm filtered subsamples from each batch of newly collected Magela Creek water were collected for analysis of total and dissolved organic carbon (Shimadzu TOC-V CSH), and alkalinity (APHA 2017; method 2320B; Envirolab). Throughout each experiment, pH, electrical conductivity (EC), and dissolved oxygen (DO) were measured on subsamples of new water (taken from each test bottle), and of old test solutions (taken from a pooled sample of each test replicate) at the start and end of tests, and during 48-h water changes (WTW Multi 340i meter, using probes: Sentix 41 for pH, Orion 013005MD for EC and CellOx 325 for DO). Incubator temperature was monitored at 5 min intervals throughout each test using remote logging equipment and software (Testo Saveris™).

Subsamples of test control waters, Milli-Q blanks, and procedural Milli-Q blanks (prepared in the same manner as the test solutions) were taken at the start of each test, filtered (0.45 µm, Minisart RC25, Sartorius Stedim), acidified (1% HNO3) and analysed for a standard suite of metals and major ions (Al, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Na, Ni, Pb, Se, SO_4, U and Zn) using inductively coupled plasma–atomic emission spectroscopy and

inductively coupled plasma–mass spectrometry at a NATA accredited laboratory (Envirolab, Chatswood NSW). Filtered subsamples of all other test treatments were taken at the start of all acute and chronic tests, and at the end of the majority of chronic tests, and analysed to determine measured Mg and Ca concentrations.

Table 5.2: Details of the 24-h acute and 14-d chronic toxicity test protocols used for MgSO4 toxicity testing.

Test criteria	**Acute toxicity test**	**Chronic toxicity test**
Test organism species	*Velesunio* spp.	*Velesunio* spp.
Life stage	Glochidia <24-h old post-release from adult female	Juvenile <24-h old post-excystment
Toxicity test type	Static renewal	Static renewal
Test duration	24 h	14 d
Test vessels	9-cm plastic petri dishes with lids	6-cm plastic jars (175 mL) with lids
Test solution volume	30 mL	100 mL
# of test organisms per vessel	10	10
# of replicates per concentration	3	2
Dilution water	Natural Magela Creek Water	Natural Magela Creek Water
Test solution renewal	none	100% every 2 d
Feeding	none	*Chlorella* algae (~7.0 - 8.0 × 10^4 cells/mL) every 2 d
Aeration	none	none
Turbidity at start and at water changes	none	100 NTU using fine sediment ≤63 μm
Assessment endpoint	Survival as measured by valve closure response after exposure to NaCl solution	Growth rate, survival
Test acceptability	Survival >90% in each control treatment	Survival >80% in each control treatment
Temperature	27.5 ± 1°C	27.5 ± 1°C
pH	6.0 ± 0.3	6.0 ± 0.3
pH control	1 mM HEPES buffer adjusted to pH 6.0	1 mM HEPES buffer adjusted to pH 6.0
Dissolved oxygen	80-120% saturation	80-120% saturation
Photoperiod	12:12-h (light: dark)	12:12-h (light: dark)

5.2.7 Test acceptability criteria

Test acceptability criteria were met if: ≥ 90% and ≥ 80% of control organisms had survived at the end of each acute and chronic test, respectively; minimum juvenile control growth was between 13–32 µm/day for chronic tests (based on mean control growth rates of 21.2 µm/day ± 5.8 standard deviation; n = 18, obtained in Kleinhenz *et al*. 2019a); average changes in pH between new and old waters did not exceed ± 0.3 units; average changes in electrical conductivity between new and old waters did not exceed 10%; dissolved oxygen remained above 80%; and average incubator temperature remained within 1°C of the target temperature. Measured Mg:Ca ratios of treatments were considered acceptable if the majority of treatments were within 80% of the target ratio of 9:1.

5.2.8 Data analysis

Measured magnesium exposure concentrations from the start of acute tests, and mean measured concentrations from the start and end of chronic tests, were used in final analyses. Survival data from acute tests, and growth rate data (expressed as percentage of the control growth rate) from chronic tests, were used for statistical analyses. Concentration-response modelling and estimation of acute median lethal concentrations (LC50s) or chronic 10% and 50% effect concentrations (EC10s and EC50s), together with their 95% confidence intervals, were conducted for each test, using non-linear, 3-parameter logistic regression (CETIS™ v1.9.0.9, Tidepool Scientific, LLC). Differences in the acute LC50s and chronic EC50s were assessed based on overlapping 95% confidence intervals. Geometric means were also calculated from the acute and chronic toxicity estimates to represent the mean toxicity values (LC50s, EC50s, and EC10s) for each species.

5.3 Results and discussion

5.3.1 Quality assurance / quality control

All tests met control performance and water quality test acceptability criteria. For acute tests, mean control survival was 99.2 ± 2.4%, pH 6.05 ± 0.02, control EC 15.6 ± 1.7 µS/cm, mean EC shift 1.9 ± 1.6%, DO 104.8 ± 5.9% and temperature 27.1 ± 0.3 °C (Tables 5.1 and 5.3). For chronic tests, mean control growth rate was 22.8 ± 2.7 µm/day, control survival was 96.7 ± 4.1%, pH 6.05 ± 0.02, control EC 16.2 ± 1.9 µS/cm, mean EC shift 0.9 ± 0.7%, DO 109.6 ± 4.7% and temperature 27.0 ± 0.2 °C (Tables 5.1 and 5.4).

The composition (alkalinity, hardness and dissolved organic carbon) of Magela Creek water was considered representative of the typically low concentrations of the creek's surface waters (Section 5.2.1, and Table 5.1). Higher concentrations of SO_4 were present due to the addition of the HEPES buffer, but were considered to have a negligible effect on Mg toxicity, based on previous research (van Dam *et al*. 2010). Chemical analyses of blank, procedural blank, and control samples from each acute and chronic toxicity test indicated that no confounding metal contamination was present except for some elevated levels of Zn (5.4–6.6 µg/L) in the control waters of four acute tests, and elevated Al (32 and 93 µg/L) in two acute tests. (Supplementary Data, Tables S5.1 and S5.2). The elevated Zn concentrations arose from sampling tubes rather than contamination of the test system. Other freshwater mussel studies have reported Zn 24-h LC50s ranging from 107 to 163 µg/L (Markich 2017), and NOEC (no observed effect concentration) values ranging from 161 to 240 µg/L (Clearwater *et al*. 2014) in low-hardness waters, indicating that Zn contamination of up to 7 µg/L in the test system probably had negligible effects. No influence of elevated Zn concentrations was observed on the toxicity of $MgSO_4$ or the performance of the controls. Elevated Al concentrations occur naturally in Magela Creek water during the wet season, and a previous study with *Velesunio* spp. reported a concentration of 96 µg/L in some control waters (Kleinhenz *et al*. 2018), which was not thought to have had significant toxic effects on glochidia. This assumption (as in the present study) was based on the acceptable control performance achieved.

Table 5.3: Mean physico-chemical variables of test solutions across all definitive 24-h acute $MgSO_4$ tests (± standard deviation).

Test	Date	Species	Site	Control survival (%)	pH (Units) [a]	Temp. (°C)	EC shift (%) [a]	Dissolved Oxygen (%) [a]	Average Mg: Ca Ratio
1	5/05/2018	*Velesunio angasi*	NC	100	6.04 ± 0.04	26.6	1.0 ± 1.1	102.9 ± 7.4	9.2:1
2	2/06/2018	*Velesunio angasi*	SB	100	6.06 ± 0.05	27.1	1.8 ± 2.3	105.7 ± 6.2	9.2:1
3	3/06/2018	*Velesunio angasi*	SB	100	6.06 ± 0.07	27.0	0.8 ± 0.6	108.4 ± 8.2	9.2:1
4	25/10/2017	*Velesunio angasi*	MB	100	6.06 ± 0.08	27.4	1.4 ± 1.6	110.5 ± 5.2	8.8:1
5	9/02/2018	*Velesunio angasi*	LB	100	6.05 ± 0.07	27.5	1.6 ± 2.3	102.8 ± 6.3	7.8:1
6	13/12/2017	*Velesunio* sp.	MC	93.3	6.00 ± 0.04	27.1	2.3 ± 1.4	91.2 ± 4.2	9.1:1
7	26/05/2018	*Velesunio* sp.	MC	100	6.08 ± 0.07	27.2	0.8 ± 0.8	105.3 ± 7.1	8.9:1
8	27/05/2018	*Velesunio* sp.	MC	100	6.06 ± 0.06	27.2	5.6 ± 3.7	105.4 ± 4.9	8.9:1
		Mean ± SD		99.2 ± 2.4	6.05 ± 0.02	27.1 ± 0.3	1.9 ± 1.6	104.8 ± 5.9	8.9:1 ± 0.5

[a] Values represent measurements of 3 replicate samples at 0 and 24 h from all treatments from each test (n = 8 per test).

NC = Nourlangie Creek, SB = Sandy Billabong, MB = Mudginberri Billabong, LB = Lake Bennett, MC = Magela Creek

Table 5.4: Mean physico-chemical variables of test solutions across all definitive 14-d chronic $MgSO_4$ tests (± standard deviation).

Test	Date	Species	Site	Control survival (%)	Control growth (µm/day)	pH (Units) [a]	Temp. (°C)	EC shift (%) [a]	Dissolved Oxygen (%) [a]	Average Mg: Ca Ratio
1	14/05/2018	*Velesunio angasi*	NC	95	20.0	6.01 ± 0.10	26.8	0.9 ± 0.4	109.4 ± 8.6	9.3:1
2	30/10/2017	*Velesunio angasi*	SB	100	21.2	6.06 ± 0.07	27.2	0.2 ± 0.3	116.5 ± 6.4	8.5:1
3	13/06/2018	*Velesunio angasi*	SB	90	21.8	6.06 ± 0.06	27.1	0.7 ± 0.5	112.8 ± 10.5	9.5:1
4	21/12/2017	*Velesunio* sp.	MC	100	22.6	6.05 ± 0.07	27.1	2.4 ± 2.2	104.8 ± 8.1	8.5:1
5	5/06/2018	*Velesunio* sp.	MC	100	27.7	6.06 ± 0.05	27.2	0.7 ± 0.4	110.0 ± 9.1	9.2:1
6	28/04/2018	*Velesunio* sp.	GC	95	23.3	6.03 ± 0.05	26.6	0.8 ± 0.8	104.2 ± 9.8	9.1:1
		Mean ± SD		96.7 ± 4.1	22.8 ± 2.7	6.05 ± 0.02	27.0 ± 0.2	0.9 ± 0.7	109.6 ± 4.7	9.0:1 ± 0.4

[a] Values represent measurements of 2 replicate samples at 0, 2, 4, 6, 8, 10, 12, 14 d from all treatments from each test (n = 8 per test).

NC = Nourlangie Creek, SB = Sandy Billabong, MC = Magela Creek, GC = Gulungul Creek

For acute tests, average measured Mg concentrations remained within 5.3% of nominals, and average measured Ca concentrations remained within 8.9% of nominals (Supplementary Data, Table S5.3). For chronic tests, average measured Mg concentrations between the start and end of tests remained within 5.8% and 6.1% of nominals respectively, and average measured Ca concentrations between the start and end of tests remained within 5.5% and 3.3% of nominals respectively (Supplementary Data, Table S5.4). Additional samples of the total Mg and Ca fractions were measured at the start and end of one chronic test, showing minimal differences in Mg or Ca concentrations between the total or dissolved fractions (Supplementary Data, Table S5.5). The measured ratio of Mg:Ca for test treatments averaged 8.9:1 (range 7.8–9.2:1) for acute tests, and 9:1 (range 8.5–9.5:1) for chronic tests.

5.3.2 Magnesium toxicity

The individual 24-h LC50s for the five acute tests with *V. angasi* ranged from 194 to 393 mg/L and were not significantly different from each other, apart from Test 3 using Sandy Billabong glochidia (Table 5.5). The LC50s for the three acute tests with *Velesunio* sp. from Magela Creek ranged from 246 to 390 mg/L, and Test 7 was significantly different from Tests 6 and 8 based on non-overlapping 95% confidence intervals (Table 5.5). The geometric means of the LC50s for each species were very similar (284 and 300 mg/L for *V. angasi* and *Velesunio* sp. respectively), indicating low inter-species variability (Table 5.5, Figure 5.2). The individual 14-d EC50s and EC10s for the three chronic tests with *V. angasi* ranged from 204 to 304 mg/L and 34 to 193 mg/L respectively (Table 5.6). Significant difference was seen between the EC50s of the two tests using Sandy Billabong juveniles (Tests 2 and 3, Table 5.6). The EC50s and EC10s for the three chronic tests with *Velesunio* sp. ranged from 205 to 282 mg/L and 67 to 117 mg/L respectively, and significant difference was seen between the EC50s of Test 5 (Magela Creek) and Test 6 (Gulungul Creek) based on non-overlapping 95% confidence intervals. The geometric means of the EC50s and EC10s also indicated low inter-species variability, with the mean EC50 and EC10 for *V. angasi* (241 and 88 mg/L) similar to those for *Velesunio* sp. (232 and 87mg/L), although individual EC10s were more variable than EC50s (Table 5.6, Figure 5.3). This concurs with previous acute and chronic ammonia toxicity studies with *V. angasi* and *Velesunio* sp., which reported low (≤2-

fold) inter-species variability (Kleinhenz *et al*. 2018; Kleinhenz *et al*. 2019a). Although data for Mg toxicity are limited for freshwater mussels, the inter-species variability of this study is comparable or lower than previous studies assessing glochidia and juveniles with other metals and salts. For example, in a toxicity database compiled by Raimondo *et al*. (2016), the mean inter-species variability in sensitivity for a range of chemicals was 2.2-fold for glochidia (n = 32), and 1.9-fold for juveniles (n = 49). Similarly, Wang *et al*. (2017) reported a mean inter-species variability of ~2-fold for juveniles of 5 mussel species in 96-h toxicity tests with chloride, potassium, SO_4 and Zn. In contrast, 13-fold inter-species variability was observed for glochidia of 4 mussel species tested in acute 24-h tests with chloride by Gillis (2011).

In the present study, intra-species variation in sensitivity in both acute and chronic tests was lower in most cases than that reported in other freshwater mussel studies. The LC50s for the five acute tests with *V. angasi* varied by 2.0-fold (194–393 mg/L Mg), while for *Velesunio* sp. the LC50s for the three acute tests varied by 1.6-fold (246–390 mg/L Mg; Table 5.5). The EC50s for the three chronic tests with *V. angasi* varied by 1.5-fold (204–304 mg/L Mg), while the EC10s varied by 5.7-fold (34–193 mg/L Mg). For *Velesunio* sp., the EC50s for the three chronic tests varied by 1.4-fold (205–282 mg/L Mg), while the EC10s varied by 1.7-fold (67–117 mg/L; Table 5.6). In comparison, intra-species variation in sensitivity for two mussel species ranged between 1 to 3-fold in acute tests (n = 66) with copper, ammonia and chlorine (Wang *et al*. 2007a), and intra-species variations of 2.5 to 8-fold were observed for four mussel species assessed with sodium chloride (Gillis 2011). Common sources of intra-species variation among toxicity estimates are generally attributed to measurement errors, experimental design, genetic variation, and phenotypic plasticity (Nikinmaa and Anttila 2019).

Although the inter- and intra-species variability in the present study was lower compared to other studies, several factors may have influenced the variability, such as seasonal or field site variations among adult mussels, and differences in the water chemistry of the diluent water used (hardness, alkalinity, dissolved organic matter). Seasonal variations in mussel sensitivity were not apparent for acute or chronic toxicity tests, with a similar range of toxicity values recorded for tests conducted in the wet season (~November–April), compared

to those conducted in the dry season (~May–October; Tables 5.5 and 5.6). Variations also occurred between and within field sites, but with no apparent bias. For example, the two acute Sandy Billabong tests with *V. angasi* varied by 1.5-fold (Tests 2 and 3; Table 5.5), and the three acute Magela Creek tests with *Velesunio* sp. (Tests 6–8; Table 5.5) varied by an average 1.4-fold (1.1 – 1.6). The variation in sensitivity between the sites was similar, varying by an average 1.4-fold (1.1 – 2.1). Similar variability was seen for chronic tests, with no clear evidence of greater sensitivity between or within field sites (Table 5.6). Differences in sensitivity were seen between acute and chronic tests using glochidia and juveniles originating from the same mussels, but the variation was inconsistent. For example, Nourlangie Creek glochidia produced the least sensitive acute results but the juveniles arising from the same brood of glochidia produced the most sensitive chronic results (Test 1; Tables 5.5 and 5.6), while Sandy Billabong glochidia produced the most sensitive acute results and the juveniles from the same brood produced the least sensitive chronic results (Test 3; Table 5.5 and 5.6).

Differences in the health of different batches of glochidia or juvenile mussels could affect sensitivity. However, the high control survival in all tests and similar control growth rate in chronic tests indicated an overall consistency in health. An increase in sensitivity was evident in two sets of acute tests that were conducted on successive days using mussels from Magela Creek, and mussels from Sandy Billabong (Table 5.5). Although these successive tests used glochidia from different mussels, the glochidia extraction took longer for each successive test, with the adult mussels held in shallow containers for two nights. Stress to the adult mussels may have weakened the glochidia. However, glochidia viability remained high (≥ 90%) for the successive tests, and the differences in sensitivity could be simply due to genetic variation, or a combination of the different factors. To minimise possible effects on glochidia sensitivity in tests carried out on successive days, the test protocol could be modified for future tests to only use glochidia released within the first 24-h.

Acute-to-chronic ratios (ACRs) can be used in ecological risk assessment where acute data are available and chronic data are limited or absent (Raimondo *et al.* 2007). Reliance on ACRs is generally not recommended as the range of reported ACRs is so great (Raimondo *et al*. 2007), and different modes of action have been observed for some chemicals in acute and

chronic toxicity tests (May *et al*. 2016). For example, an evaluation of 456 aquatic invertebrate and fish ACRs resulted in a median ACR of 8.3, but the range of ACRs was extremely high (between 1 and >10,000), and was attributed to differences in chemical modes of action, taxa, and ambient habitat media (Raimondo *et al*. (2007).

Studies using temperate mussel species have reported ACRs ranging from 11 to 18 for ammonia (Wang *et al*. 2007c), 3.2 for sulfate (Wang *et al*. 2016), and 3.2 and 4.1 to 4.7 for copper (March *et al*. 2007; Wang *et al*. 2007c respectively), highlighting that ACRs vary between different mussel species and contaminants, and that their use for deriving GVs may add significant uncertainty. However, the ACR (geometric mean of all LC50s/EC10s) of 3.3 for *Velesunio* spp. exposed to Mg in the present study was similar to the ACR of 3.2 for *Velesunio* spp. exposed to ammonia calculated from previous studies (Kleinhenz *et al*., 2018; Kleinhenz *et al*. 2019a). These values derived using the same test methods were more consistent, suggesting that the ACRs could be applied to acute toxicity data to estimate chronic toxicity for *Velesunio* spp. for these contaminants. However, more research would be needed to determine the consistency of ACRs for *Velesunio* spp. with these and other contaminants, before applying them to derive data for GVs and environmental management.

5.3.3 Data comparisons

Of the data available on the ECOTOX database (https://cfpub.epa.gov/ecotox/) and other studies for freshwater taxa with Mg or Mg compounds (e.g. MgCl, $MgSO_4$, $MgNO_3$), valid toxicity data (reporting measured Mg concentrations and relevant endpoints) that could be used for comparison with LC50, EC10 and EC50 data from this study were limited. Acute mortality data used for comparison included data from seven studies reporting LC50 toxicity estimates. Chronic data were limited to two studies reporting EC50 and EC10 toxicity estimates (Tables 5.7 and 5.8). No valid acute or chronic Mg toxicity data were available for freshwater mussels.

Table 5.5: Summary of the acute tests with *Velesunio* spp. glochidia and the $MgSO_4$ toxicity estimates (95% CI).

Test	Species	Site	Test start date	Control survival (%)	Mg:Ca ratio (measured)	LC50 (mg/L) Individual tests	LC50 (mg/L) Geometric mean
1	*Velesunio angasi*	NC	05/05/2018	100	9.2:1	393 (303 – 458)	
2	*Velesunio angasi*	SB	02/06/2018	100	9.2:1	288 (269 – 306)	
3	*Velesunio angasi*	SB	03/06/2018	100	9.2:1	194 (168 – 223)	
4	*Velesunio angasi*	MB	25/10/2017	100	8.8:1	263 (198 – 334)	
5	*Velesunio angasi*	LB	09/02/2018	100	7.8:1	320 (294 – 347)	284
6	*Velesunio* sp.	MC	13/12/2017	93.3	9.1:1	246 (89 – 357)	
7	*Velesunio* sp.	MC	26/05/2018	100	8.9:1	390 (364 – 417)	
8	*Velesunio* sp.	MC	27/05/2018	100	8.9:1	281 (254 – 308)	300

NC = Nourlangie Creek, SB = Sandy Billabong, MB = Mudginberri Billabong, LB = Lake Bennett, MC = Magela Creek

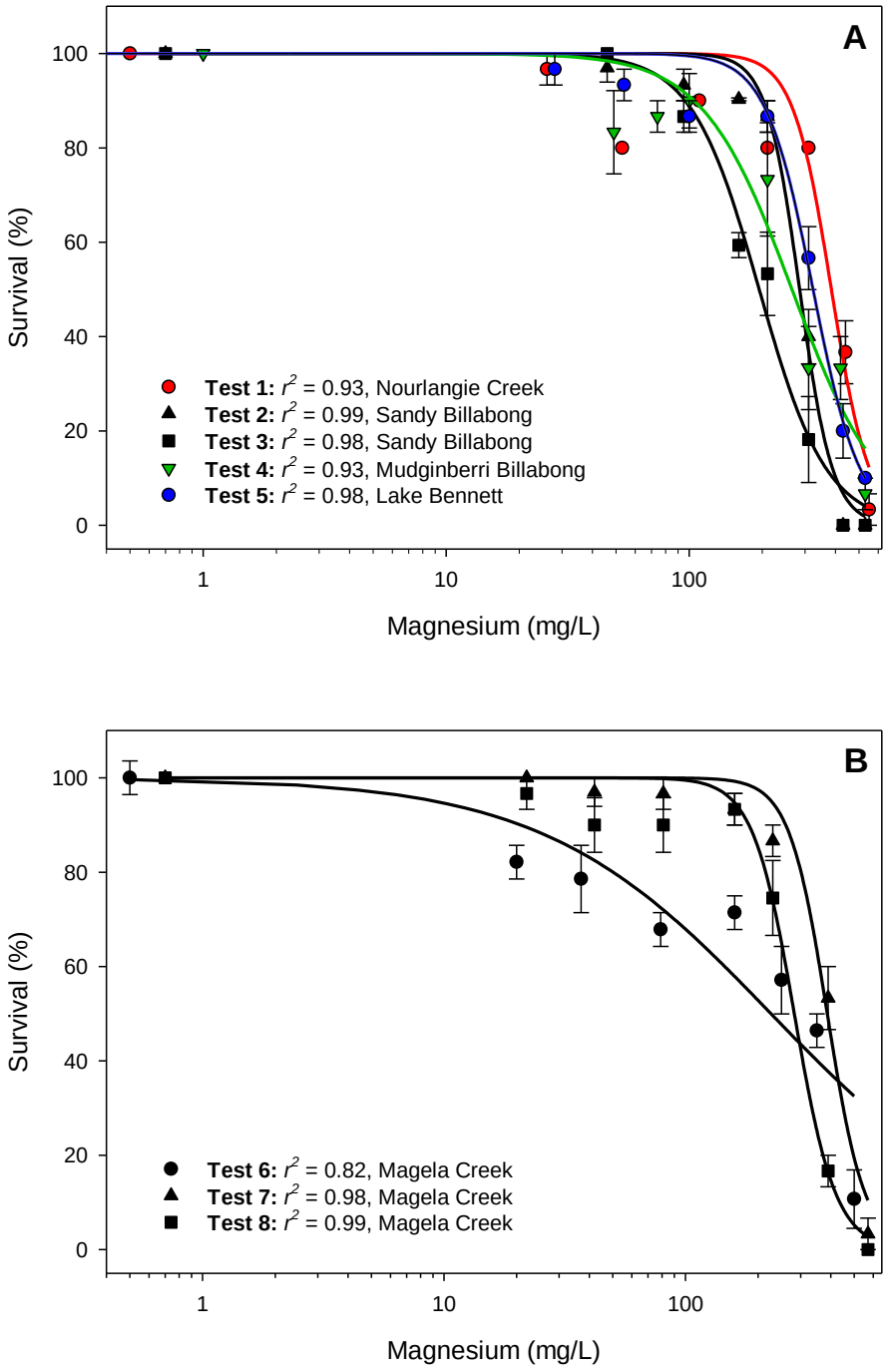

Figure 5.2: Comparison of the magnesium concentration-response relationships for 24-h acute exposures with (A) *Velesunio* sp. glochidia, and (B) *Velesunio angasi* glochidia. Data points represent the mean ± standard error of three replicates per treatment, with eight treatments per test. The 95% confidence intervals have been removed for clarity. Toxicity estimates were determined using 3-parameter logistic models.

Table 5.6: Summary of the chronic 14-d tests with *Velesunio* spp. juveniles and the $MgSO_4$ toxicity estimates (95% CI).

Test	Species	Site	Test start date	Control survival (%)	Av. Control growth rate (um/day)	Mg:Ca ratio (measured)	EC50 (mg/L) Individual tests	EC50 (mg/L) Geometric mean	EC10 (mg/L) Individual tests	EC10 (mg/L) Geometric mean
1	*Velesunio angasi*	NC	14/05/2018	95	20.0	9.3:1	204 (145 – 285)		34 (n/a – 86)	
2	*Velesunio angasi*	SB	30/10/2017	100	21.2	8.5:1	227 (202 – 256)		104 (41 – 136)	
3	*Velesunio angasi*	SB	13/06/2018	90	21.8	9.5:1	304 (275 – 337)	241	193 (59 – 230)	88
4	*Velesunio* sp.	MC	21/12/2017	100	22.6	8.5:1	216 (161 – 290)		67 (n/a – 119)	
5	*Velesunio* sp.	MC	5/06/2018	100	27.7	9.2:1	205 (194 – 216)		85 (72 – 97)	
6	*Velesunio* sp.	GC	28/04/2018	95	23.3	9.1:1	282 (250 – 318)	232	117 (84 – 143)	87

NC = Nourlangie Creek, SB = Sandy Billabong, MC = Magela Creek, GC = Gulungul Creek

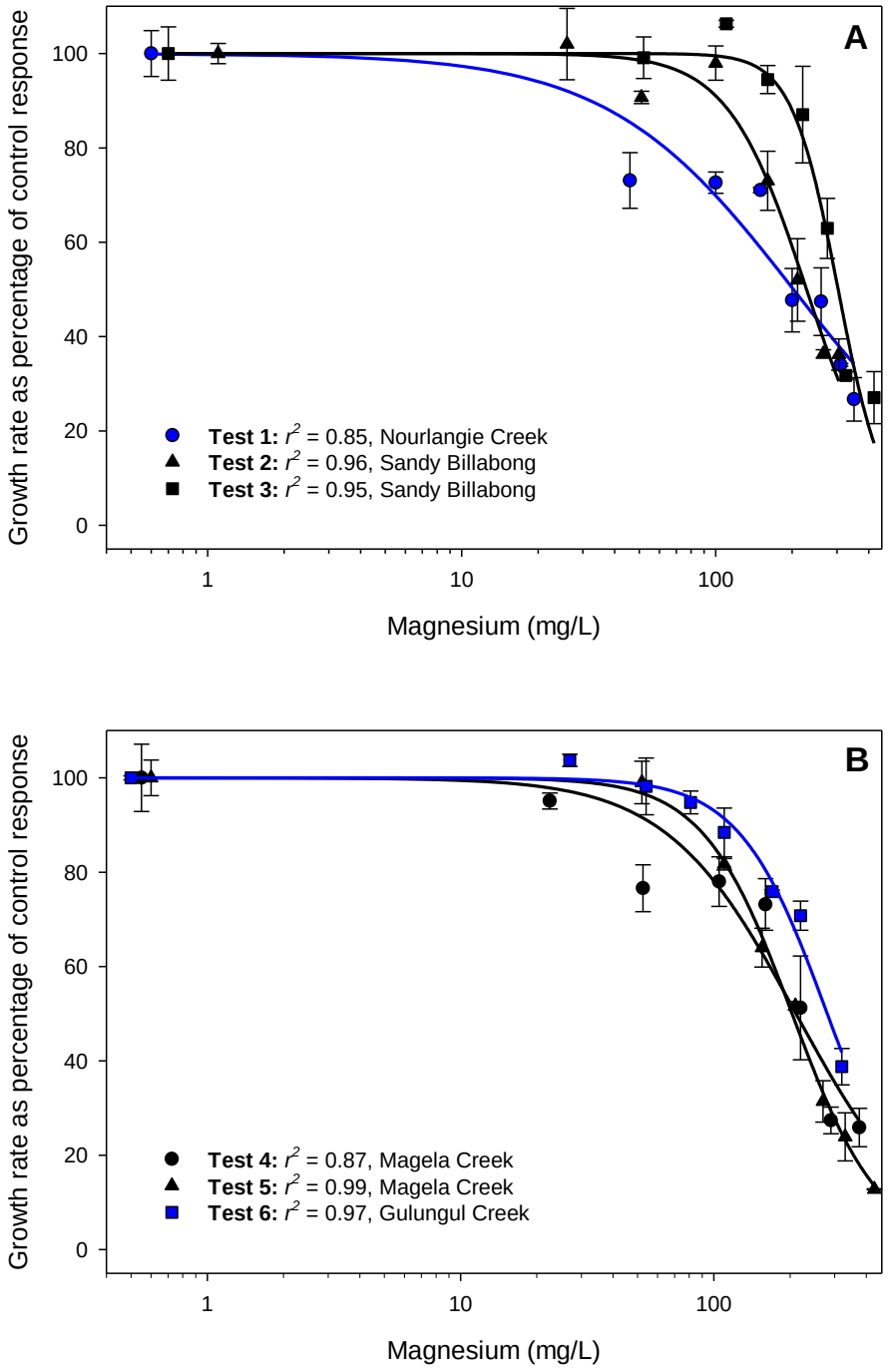

Figure 5.3: Comparison of the magnesium concentration-response relationships for 14-d chronic exposures with (A) *Velesunio* sp. juveniles, and (B) *Velesunio angasi* juveniles. Data points were transformed to percentage of the control growth rate (µm/day) and represent the mean ± standard error of two replicates per treatment, with eight treatments per test. The 95% confidence intervals have been removed for clarity. Toxicity estimates were determined using 3-parameter logistic models.

Table 5.7: Mg toxicity data from the international literature.

Species	Chemical	Test type	Magnesium concentration (mg/L)			Reference
			LC50	EC50/IC50	EC10/IC10	
Crustacean – water flea (*Ceriodaphnia dubia*)	$MgCl_2$	48-h survival	69 - 1290	-	-	Mount *et al*. (2016)
Crustacean – water flea (*Daphnia magna*)	Mg Cl_2	48-h survival (no food)	140	-	-	Biesinger and Christensen (1972)
Crustacean – water flea (*Ceriodaphnia dubia*)	$MgSO_4$	48-h survival	160 - 2860	-	-	Mount *et al*. (2016)
Mayfly (*Neocloeon triangulifer*)	$MgSO_4$	96-h survival	224 – 536			Soucek *et al.* (2018)
Crustacean – crayfish (*Austropotamobius pallipes* sp.)	Mg Cl_2	30-d survival	270 – 360	-	-	Boutet and Chaisemartin (1973)
Crustacean – water flea (*Daphnia magna*)	Mg Cl_2	48-h survival (with food)	322	-	-	Biesinger and Christensen (1972)
Crustacean – water flea (*Ceriodaphnia dubia*)	$MgCO_3$	48-h survival	390 - 977	-	-	Mount *et al*. (2016)
Crustacean – crayfish (*Orconectes limosus*)	Mg Cl_2	30-d survival	440	-	-	Boutet and Chaisemartin (1973)
Crustacean – crayfish (*Austropotamobius pallipes* sp.)	Mg Cl_2	96-h survival	480	-	-	Boutet and Chaisemartin (1973)
Crustacean – crayfish (*Orconectes limosus*)	Mg Cl_2	30-d survival	570	-	-	Boutet and Chaisemartin (1973)
Crustacean – crayfish (*Orconectes limosus*)	Mg Cl_2	96-h survival	760	-	-	Boutet and Chaisemartin (1973)
Worm (*Caenorhabditis elegans*)	Mg Cl_2	24-h survival	4389	-	-	Tatara *et al*. (1997)
Duckweed (*Lemna aequinoctialis*)	$MgSO_4$	96-h plant growth	-	4.4	1.9	van Dam *et al.* (2010)
Duckweed (*Lemna aequinoctialis*)	Mg Cl_2	96-h plant growth	-	6.8	-	van Dam *et al.* (2010)
Green hydra (*Hydra viridissima*)	$MgSO_4$	96-h population growth	-	11	1.8	van Dam *et al.* (2010)
Snail (*Amerianna cumingi*)	$MgSO_4$	96-h embryo production	-	13	4.5	van Dam *et al.* (2010)
Snail (*Amerianna cumingi*)	Mg Cl_2	96-h embryo production	-	20	-	van Dam *et al.* (2010)
Green hydra (*Hydra viridissima*)	Mg Cl_2	96-h population growth	-	27	-	van Dam *et al.* (2010)
Cladoceran (*Moinodaphnia macleayi*)	$MgSO_4$	3 brood production	-	63	36	van Dam *et al.* (2010)
Crustacean – water flea (*Daphnia magna*)	Mg Cl_2	21-d reproduction		125	-	Biesinger and Christensen (1972)
Crustacean – water flea (*Daphnia magna*)	Mg Cl_2	21-d survival	190	-	-	Biesinger and Christensen (1972)
Green alga (*Chlorella* sp.)	$MgSO_4$	72-h cell division rate	-	1215	43	van Dam *et al.* (2010)

Table 5.8: Comparison of Mg toxicity estimates from this study with other tropical species data derived using a Mg: Ca of 9:1

Species	**Common name**	**Test type**	**LC50 (mg/L)**	**EC50/IC50 (mg/L)**	**EC10/IC10 (mg/L)**	**Reference**
Amerianna cumingi	Snail	96-h embryo production		96	5.6	van Dam *et al*. (2010)
Lemna aequinoctialis	Duckweed	96-h plant growth		629	36	van Dam *et al*. (2010)
Moinodaphnia macleayi	Cladoceran	3 brood production		122	39	van Dam *et al*. (2010)
Velesunio angasi	Mussel	24-h survival / 14 d growth rate	284	241	88	This study
Velesunio sp.	Mussel	24-h survival / 14 d growth rate	300	232	87	This study
Hydra viridissima	Green hydra	96-h population growth		713	246	van Dam *et al*. (2010)
Chlorella sp.	Green alga	72-h cell division rate		3435	818	van Dam *et al*. (2010)
Mogurnda mogurnda	Fish	96-h survival - acute	4054			van Dam *et al*. (2010)

5.3.3.1 Acute toxicity

The LC50s for *Velesunio* species glochidia were lower (indicating higher sensitivity) than seven of the acute LC50s reported for other freshwater taxa (Table 5.7), although these were difficult to compare due to differences in diluent water physico-chemistry. The sensitivity of *V. angasi* and *Velesunio* sp. (LC50s 284 and 300 mg/L respectively) was comparable to that of the crayfish, *Austropotamobius pallipes* spp. (LC50s ranging 270 – 480 mg/L in 96-h and 30-d tests, Boutet and Chaisemartin 1973), while sensitivity was higher than another crayfish species, *Orconectes limosus* (LC50s ranging between 440 – 760 mg/L, Boutet and Chaisemartin 1973). Sensitivity was comparable to that of the water flea, *Daphnia magna* in 48-h tests with food (LC50 = 322 mg/L), but lower than *D. magna* in 48-h tests without food (LC50 = 140 mg/L; Biesinger and Christensen, 1972). The nematode worm, *Caenorhabditis elegans*, was the least sensitive species for which data were available (LC50 = 4,389 mg/L; Tatara *et al.* 1997). More recent studies have demonstrated increased sensitivity of freshwater organisms in waters of low hardness and high Mg:Ca ratios (i.e. lower Ca). For example, Mount *et al.* (2016) evaluated the toxicities of major ion salts for the crustacean, *Ceriodaphnia dubia*, using a range of dilution waters with differing hardness (ranging 10.6 – 160 mg $CaCO_3$), and differing Mg:Ca ratios. LC50s for $MgCl_2$, $MgSO_4$, or $MgCO_3$, ranged from 69 to 2860 mg/L Mg with the highest sensitivity found in waters with the lowest hardness (10.6 mg $CaCO_3$), or highest Mg:Ca ratio (3.7:1). Soucek *et al*. (2018) reported 96-h LC50s ranging from 224 – 536 mg/L for the mayfly (*Neocloeon triangulifer*) tested with $MgSO_4$ in dilution waters of four different hardnesses (28–212 mg/L $CaCO_3$) and two different Mg:Ca ratios (2:1 and 0.2:1 mass ratios). Again, the LC50 increased with increasing hardness, and decreased with decreasing Mg:Ca ratio, by approximately 1.5-fold. When compared to the LC50 derived in waters of the same chemistry, both *Velesunio* species were approximately 14 times more acutely sensitive than *M. mogurnda* (Table 5.8). As shown for other freshwater species, the sensitivity of *Velesunio* spp. to Mg may be increased in the very soft diluent water (hardness ~3.0 mg/L as CaCO3) from Magela Creek. Comparison with toxicity data for different taxonomic groups is problematic given the variability amongst species responses, and further data are needed for freshwater mussels in a range of water hardnesses.

5.3.3.2 Chronic toxicity

When compared to chronic data for other freshwater taxa, the EC50s and EC10s for juvenile *Velesunio* species were higher than all except one of the nine chronic EC50s reported, and higher than all of the 5 chronic EC10s reported (Table 5.7), suggesting lower sensitivity to Mg. However, as with the acute data comparisons, chronic data comparisons with other studies should be made with caution, due to differences in test conditions, endpoints, and the physico-chemistry of the test waters used. The chronic EC50s for *V. angasi* and *Velesunio* sp. (241 and 232 mg/L respectively) were higher than that of 125 mg/L reported for the water flea, *Daphnia magna,* by Biesinger and Christensen (1972). However, mean chemical characteristics of the diluent water used in that study were notably different (pH 7.74, hardness 45.3 mg/L $CaCO_3$, alkalinity 42.3 mg/L $CaCO_3$, Mg:Ca ratio of 0.22:1) to the present study, and may have influenced sensitivity. The low calcium concentration of the diluent water (Magela Creek water) used in the present study is likely to increase the sensitivity of *Velesunio* spp. to Mg as a result of increased bioavailability of the Mg ions. Previous studies have also demonstrated that diluent waters of low hardness have likely contributed to the increased sensitivity of aquatic organisms to some toxicants, such as ammonia (Mooney *et al*. 2019), sulfate (Soucek and Kennedy 2005; Davies and Hall 2007), manganese (Harford *et al*. 2015), uranium (Riethmuller *et al.* 2001; Markich 2013), and nickel (Leonard and Wood 2013). Oliveira-Filho *et al*. (2014) demonstrated a reduction in egg production for the snail, *Biomphalaria glabrata,* in waters of decreasing hardness. They noted that aquatic species become more susceptible to the action of toxicants in very soft water due to the absence of ameliorative ions. Increased bioavailability of toxic ions may also occur as a result of the reduced complexation activity in low ionic strength waters (Mount *et al*. 2016).

The toxicity estimates reported in van Dam *et al.* (2010) for other tropical freshwater taxa were more suitable for comparison with *Velesunio* spp. data from the present study, being derived using the same low ionic composition test diluent (Magela Creek water), and the same Mg:Ca ratio of 9:1. When comparing the mean chronic EC50 values for *V. angasi* and *Velesunio* sp. (241 and 232 mg/L respectively) with those reported in van Dam *et al*. (2010), *Velesunio* spp. were the third most sensitive species, being approximately 2.5-fold less sensitive than the most sensitive species, *A. cumingi,* (EC50 = 96 mg/L), and approximately 2-fold less sensitive than *Moinodaphnia macleayi* (EC50 = 122 mg/L).

Velesunio spp. were between 2.6 – 14.8-fold more sensitive than the other three species, *L. aequinoctialis* (629 mg/L), *H. viridissima,* (713 mg/L), and *Chlorella* sp., (3435 mg/L; Table 5.8). A similar order of sensitivity was apparent when comparing the mean chronic EC10 values for *V. angasi* and *Velesunio* sp. (88 and 87 mg/L) with those of van Dam *et al*. (2010), except that *L. aequinoctialis* was two to three times more sensitive (Table 5.8).

Other differences in sensitivity may be explained in part by physiological factors, such as the calcium requirements of each species. The calcium ion is a major component of adult and larval mollusc shells and soft tissue, and is also important for cellular signalling and regulation (Machado and Lopes-Lima 2011). The concentrations of calcium carbonate in freshwater snail shells have been measured using ion chromatography, and reported to comprise from 97.0 to 98.2% (White *et al*. 2007). Similarly, Ca was shown to be the most abundant element in both the shell and soft tissues of freshwater mussels, as well as aquatic plants (Ravera *et al*. 2003). van Dam *et al.* (2010) reported a much higher (>250-fold) sensitivity to Mg for the aquatic plant, *L. aequinoctialis,* than the algae, *Chlorella* sp., when tested in waters of low background Ca concentrations (< 0.8 mg/L). This difference was hypothesised to be due to the very different Ca requirements of each species, *Chlorella* being less dependent on Ca, and hence less sensitive to Mg in its capacity as a Ca channel antagonist. Calcium uptake in freshwater mussels is mainly via active transport through Ca channels. Factors influencing the uptake of calcium in mussels include the physical parameters of the water such as pH and temperature, the ionic composition of the water, toxicants or pollutants in the water, and biological factors such as parasites (Machado and Lopes-Lima 2011). As the physico-chemistry of the diluent water used in the present study was very similar amongst tests, it was not possible to investigate trends associated with changing pH, temperature, or ionic composition. Similarly, the effects on sensitivity under changing Mg:Ca ratios were not investigated during this study, but previous research has shown that, in general, higher Mg:Ca ratios (i.e. less Ca) increase the toxicity of Mg, although the strength of Ca amelioration varies among tropical freshwater species (van Dam *et al*. 2010). Additional Mg exposures to *Velesunio* spp. at a range of Mg:Ca ratios would be needed to determine the extent to which Ca has an ameliorative effect on Mg toxicity to the mussels investigated in this study.

5.4 Conclusion

This study presented the first acute and chronic magnesium toxicity estimates for tropical freshwater mussels, using recently optimised test protocols. The toxicity estimates indicated that *Velesunio angasi* and *Velesunio* sp. were moderately sensitive to Mg when compared with other tropical taxa that were tested under the same physico-chemical conditions (similar water composition and Mg:Ca ratio of 9:1). The results will contribute to the revision of a site-specific GV for Mg. Future work is recommended for assessing the effects of different Mg:Ca ratios on Mg toxicity to *Velesunio* spp.

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Supplementary Data

Table S5.1: Water chemistry measurements of blanks, pro-blanks and control treatments (Magela Creek water) for each acute test. < = below detection limit.

Test			1. Nourlangie Creek			2. Sandy Billabong			3. Sandy Billabong		
		Detection limit	BLK	PRO BLK	CONTROL	BLK	PRO BLK	CONTROL	BLK	PRO BLK	CONTROL
Date sampled			7/05/18	7/05/18	7/05/18	2/06/18	2/06/18	2/06/18	2/06/18	2/06/18	2/06/18
Date analysed			22/05/18	22/05/18	22/05/18	26/06/18	26/06/18	26/06/18	26/06/18	26/06/18	26/06/18
Aluminium	µg/L	**0.1**	0.6	0.9	17	0.9	1.3	14	0.9	1.3	14
Cadmium	µg/L	**0.02**	<	<	<	<	<	<	<	<	<
Cobalt	µg/L	**0.01**	<	<	0.05	<	<	0.06	<	<	0.06
Chromium	µg/L	**0.1**	<	<	0.1	<	<	0.1	<	<	0.1
Copper	µg/L	**0.01**	0.02	0.06	0.12	<	0.02	0.1	<	0.02	0.1
Iron	µg/L	**1**	<	<	71	<	<	61	<	<	61
Manganese	µg/L	**0.01**	<	<	1.2	<	<	1.7	<	<	1.7
Nickel	µg/L	**0.01**	0.02	0.06	0.18	0.02	0.05	0.63	0.02	0.05	0.63
Lead	µg/L	**0.01**	0.02	0.03	0.04	0.02	0.02	0.04	0.02	0.02	0.04
Selenium	µg/L	**0.2**	<	<	<	<	<	<	<	<	<
Uranium	µg/L	**0.001**	<	0.001	0.13	0.002	0.005	0.16	0.002	0.005	0.16
Zinc	µg/L	**0.1**	<	0.2	0.5	0.2	0.3	0.7	0.2	0.3	0.7
Calcium	mg/L	**0.1**	<	<	<	<	<	0.1	<	<	0.1
Magnesium	mg/L	**0.1**	<	<	0.5	<	<	0.7	<	<	0.7
Sodium	mg/L	**0.1**	<	<	1.4	<	<	1.7	<	<	1.7
Sulphate	mg/L	**0.5**	<	<	94	<	<	92	<	<	92

Table S5.1 (continued)…

Test			4. Mudginberri Billabong			5. Lake Bennett		
		Detection limit	BLK	PRO BLK	CONTROL	BLK	PRO BLK	CONTROL
Date sampled			25/10/17	25/10/17	25/10/17	9/02/18	9/02/18	9/02/18
Date analysed			22/11/17	22/11/17	22/11/17	16/02/18	16/02/18	16/02/18
Aluminium	µg/L	**0.1**	0.1	0.5	10	0.6	0.6	93
Cadmium	µg/L	**0.02**	<	<	<	<	<	0.02
Cobalt	µg/L	**0.01**	<	<	0.05	<	<	0.08
Chromium	µg/L	**0.1**	<	<	<	<	<	0.2
Copper	µg/L	**0.01**	0.01	0.15	0.25	0.03	0.06	0.18
Iron	µg/L	**1**	<	<	63	<	<	57
Manganese	µg/L	**0.01**	<	0.01	3	<	<	2.6
Nickel	µg/L	**0.01**	0.02	0.06	0.15	0.02	0.27	2.6
Lead	µg/L	**0.01**	<	<	0.07	0.03	0.04	0.14
Selenium	µg/L	**0.2**	<	<	<	<	<	<
Uranium	µg/L	**0.001**	0.007	0.009	0.031	<	0.001	0.019
Zinc	µg/L	**0.1**	<	1.1	5.4	<	<	1.5
Calcium	mg/L	**0.1**	<	<	0.2	<	<	<
Magnesium	mg/L	**0.1**	<	<	1	<	<	0.2
Sodium	mg/L	**0.1**	<	<	1.9	<	<	2.4
Sulphate	mg/L	**0.5**	<	<	120	<	<	94

Table S5.1 (continued)…

Test			6. Magela Creek			7. Magela Creek			8. Magela Creek		
		Detection limit	BLK	PRO BLK	CONTROL	BLK	PRO BLK	CONTROL	BLK	PRO BLK	CONTROL
Date sampled			13/12/17	13/12/17	13/12/17	26/05/18	26/05/18	26/05/18	26/05/18	26/05/18	26/05/18
Date analysed			11/01/18	9/01/18	9/01/18	26/06/18	26/06/18	26/06/18	26/06/18	26/06/18	26/06/18
Aluminium	µg/L	**0.1**	0.6	0.3	32	0.7	0.6	16	0.7	0.6	16
Cadmium	µg/L	**0.02**	<	<	0.03	<	<	<	<	<	<
Cobalt	µg/L	**0.01**	<	<	0.08	<	<	0.06	<	<	0.06
Chromium	µg/L	**0.1**	<	<	0.1	<	<	0.2	<	<	0.2
Copper	µg/L	**0.01**	0.03	0.04	0.41	<	0.05	0.62	<	0.05	0.62
Iron	µg/L	**1**	<	<	48	<	<	61	<	<	61
Manganese	µg/L	**0.01**	<	<	3	<	<	1.5	<	<	1.5
Nickel	µg/L	**0.01**	0.03	0.04	0.3	0.04	0.07	1	0.04	0.07	1
Lead	µg/L	**0.01**	0.03	0.04	0.32	0.02	0.02	0.13	0.02	0.02	0.13
Selenium	µg/L	**0.2**	<	<	<	<	<	<	<	<	<
Uranium	µg/L	**0.001**	<	<	0.022	0.014	0.001	0.03	0.014	0.001	0.03
Zinc	µg/L	**0.1**	<	<	6.6	0.1	0.2	6.1	0.1	0.2	6.1
Calcium	mg/L	**0.1**	<	<	0.2	<	<	0.2	<	<	0.2
Magnesium	mg/L	**0.1**	<	<	0.5	<	<	0.7	<	<	0.7
Sodium	mg/L	**0.1**	<	<	2	<	<	1.7	<	<	1.7
Sulphate	mg/L	**0.5**	<	<	84	<	<	87	<	<	87

Table S5.2: Water chemistry measurements of blanks, pro-blanks and control treatments (Magela Creek water) for each chronic test (< = below detection limit).

Test			1. Nourlangie Creek			2. Sandy Billabong			3. Sandy Billabong		
		Detection limit	BLK	PRO BLK	CONTROL	BLK	PRO BLK	CONTROL	BLK	PRO BLK	CONTROL
Date sampled			14/05/18	14/05/18	14/05/18	30/10/17	30/10/17	30/10/17	12/06/18	13/06/18	13/06/18
Date analysed			1/06/18	1/06/18	1/06/18	22/11/17	22/11/17	22/11/17	17/07/18	17/07/18	17/07/18
Aluminium	µg/L	**0.1**	0.2	0.2	16	0.1	0.4	14	0.4	0.4	13
Cadmium	µg/L	**0.02**	<	<	<	<	<	<	<	<	<
Cobalt	µg/L	**0.01**	<	<	0.18	<	<	0.11	<	<	0.06
Chromium	µg/L	**0.1**	<	<	0.1	<	<	<	<	<	0.1
Copper	µg/L	**0.01**	<	0.02	0.13	0.05	0.04	0.18	<	0.01	0.09
Iron	µg/L	**1**	<	<	120	<	<	87	<	<	34
Manganese	µg/L	**0.01**	<	<	7.5	<	<	8.5	<	<	3.7
Nickel	µg/L	**0.01**	0.02	0.04	0.17	<	<	0.1	0.06	0.06	0.28
Lead	µg/L	**0.01**	0.02	0.02	0.03	<	<	0.04	0.02	0.02	0.04
Selenium	µg/L	**0.2**	<	<	<	<	<	<	<	<	<
Uranium	µg/L	**0.001**	<	<	<	0.006	0.006	0.02	0.002	0.002	0.01
Zinc	µg/L	**0.1**	<	<	0.3	<	<	0.7	<	<	0.6
Calcium	mg/L	**0.1**	<	<	0.1	<	<	0.2	<	<	0.1
Magnesium	mg/L	**0.1**	<	<	0.6	<	<	0.9	<	<	0.7
Sodium	mg/L	**0.1**	<	<	1.6	<	<	1.8	<	<	1.8
Sulphate	mg/L	**0.5**	<	<	92	<	<	95	<	<	95

Table S5.2 (continued)…

Test			4. Magela Creek			5. Magela Creek			6. Gulungul Creek		
		Detection limit	BLK	PRO BLK	CONTROL	BLK	PRO BLK	CONTROL	BLK	PRO BLK	CONTROL
Date sampled			21/12/17	21/12/17	21/12/17	5/06/18	5/06/18	5/06/18	28/04/18	28/04/18	28/04/18
Date analysed			16/01/18	16/01/18	16/01/18	26/06/18	26/06/18	26/06/18	15/05/18	15/05/18	15/05/18
Aluminium	µg/L	**0.1**	0.5	0.6	34	0.7	1.2	17	0.5	1.4	16
Cadmium	µg/L	**0.02**	<	<	<	<	<	<	<	<	<
Cobalt	µg/L	**0.01**	<	<	0.11	<	<	0.08	<	<	0.13
Chromium	µg/L	**0.1**	<	<	0.2	<	<	0.1	<	<	<
Copper	µg/L	**0.01**	0.02	0.02	0.17	<	0.02	0.11	0.01	0.27	0.19
Iron	µg/L	**1**	<	<	90	<	<	49	<	<	130
Manganese	µg/L	**0.01**	<	0.06	6.3	<	<	4.7	<	0.04	6.5
Nickel	µg/L	**0.01**	0.04	0.05	0.2	0.02	0.04	0.13	0.02	0.22	0.25
Lead	µg/L	**0.01**	0.03	0.02	0.05	0.02	0.02	0.03	0.02	0.03	0.04
Selenium	µg/L	**0.2**	<	<	<	<	<	<	<	<	<
Uranium	µg/L	**0.001**	<	<	0.022	0.002	0.002	0.019	<	0.001	0.012
Zinc	µg/L	**0.1**	<	<	0.4	0.1	0.3	0.3	<	0.6	0.5
Calcium	mg/L	**0.1**	<	<	0.2	<	<	0.1	<	<	<
Magnesium	mg/L	**0.1**	<	<	0.5	<	<	0.6	<	0.2	0.5
Sodium	mg/L	**0.1**	<	<	1.9	<	<	1.7	<	<	2
Sulphate	mg/L	**0.5**	<	<	92	<	<	89	<	1	95

Table S5.3: Nominal and measured magnesium and calcium concentrations (mg/L) for all acute Mg tests. MC = Magela Creek, NC = Nourlangie Creek, SB = Sandy Billabong, MB = Mudginberri Billabong, LB = Lake Bennett.

Test/Site	Treatment	Magnesium (mg/L)			Calcium (mg/L)			Mg: Ca Ratio
		Nominal	Measured (start only)	% difference Measured/nominal	Nominal	Measured (start only)	% difference Measured/nominal	
1. NC	A - Control	0	0.5		0.00	<0.1		5.00
	B	25	26	4.00	2.78	2.9	4.40	8.97
	C	50	53	6.00	5.56	5.4	-2.80	9.81
	D	100	110	10.00	11.11	11	-1.00	10.00
	E	200	210	5.00	22.22	21	-5.50	10.00
	F	300	310	3.33	33.33	33	-1.00	9.39
	G	400	440	10.00	44.44	42	-5.50	10.48
	H	500	550	10.00	55.56	54	-2.80	10.19
2. SB	A - Control	0	0.7		0.00	0.1		7.00
	B	50	46	-8.00	5.56	5.5	-1.00	8.36
	C	100	95	-5.00	11.11	11	-1.00	8.64
	D	150	160	6.67	16.67	16	-4.00	10.00
	E	200	210	5.00	22.22	22	-1.00	9.55
	F	300	310	3.33	33.33	32	-4.00	9.69
	G	400	430	7.50	44.44	43	-3.25	10.00
	H	500	530	6.00	55.56	51	-8.20	10.39

Table S5.3 (continued)…

Test/Site	Treatment	Magnesium (mg/L)			Calcium (mg/L)			Mg: Ca Ratio
		Nominal	Measured (start only)	% difference Measured/nominal	Nominal	Measured (start only)	% difference Measured/nominal	
3. SB	A - Control	0	0.7		0.00	0.1		7.00
	B	50	46	-8.00	5.56	5.5	-1.00	8.36
	C	100	95	-5.00	11.11	11	-1.00	8.64
	D	150	160	6.67	16.67	16	-4.00	10.00
	E	200	210	5.00	22.22	22	-1.00	9.55
	F	300	310	3.33	33.33	32	-4.00	9.69
	G	400	430	7.50	44.44	43	-3.25	10.00
	H	500	530	6.00	55.56	51	-8.20	10.39
4. MB	A - Control	0	1		0.00	0.2		5.00
	B	50	49	-2.00	5.56	6	8.00	8.17
	C	75	74	-1.33	8.33	5.4	-35.20	13.70
	D	100	100	0.00	11.11	12	8.00	8.33
	E	200	210	5.00	22.22	23	3.50	9.13
	F	300	310	3.33	33.33	34	2.00	9.12
	G	400	420	5.00	44.44	49	10.25	8.57
	H	500	530	6.00	55.56	65	17.00	8.15

Table S5.3 (continued)…

Test/Site	Treatment	Magnesium (mg/L)			Calcium (mg/L)			Mg: Ca Ratio
		Nominal	Measured (start only)	% difference Measured/nominal	Nominal	Measured (start only)	% difference Measured/nominal	
5. LB	A - Control	0	0.2		0.00	<0.1		2.00
	B	25	28	12.00	2.78	7.4	166.40	3.78
	C	50	54	8.00	5.56	5.8	4.40	9.31
	D	100	100	0.00	11.11	11	-1.00	9.09
	E	200	210	5.00	22.22	22	-1.00	9.55
	F	300	310	3.33	33.33	33	-1.00	9.39
	G	400	430	7.50	44.44	45	1.25	9.56
	H	500	530	6.00	55.56	55	-1.00	9.64
6. MC	A - Control	0	0.5		0	0.2		2.50
	B	20	20	0.00	2.22	2	-10.00	10.00
	C	40	37	-7.50	4.44	3.8	-14.50	9.74
	D	80	79	-1.25	8.89	7.4	-16.75	10.68
	E	160	160	0.00	17.78	15	-15.63	10.67
	F	240	250	4.17	26.67	24	-10.00	10.42
	G	360	350	-2.78	40.00	36	-10.00	9.72
	H	500	500	0.00	55.56	53	-4.60	9.43

Table S5.3 (continued)…

Test/Site	Treatment	Magnesium (mg/L)			Calcium (mg/L)			Mg: Ca Ratio
		Nominal	Measured (start only)	% difference Measured/nominal	Nominal	Measured (start only)	% difference Measured/nominal	
7. MC	A - Control	0	0.7		0.00	0.2		3.50
	B	20	22	10.00	2.22	2.4	8.00	9.17
	C	40	42	5.00	4.44	4.3	-3.25	9.77
	D	80	81	1.25	8.89	9.7	9.12	8.35
	E	160	160	0.00	17.78	16	-10.00	10.00
	F	240	230	-4.17	26.67	26	-2.50	8.85
	G	360	390	8.33	40.00	39	-2.50	10.00
	H	500	570	14.00	55.56	51	-8.20	11.18
8. MC	A - Control	0	0.7		0.00	0.2		3.50
	B	20	22	10.00	2.22	2.4	8.00	9.17
	C	40	42	5.00	4.44	4.3	-3.25	9.77
	D	80	81	1.25	8.89	9.7	9.12	8.35
	E	160	160	0.00	17.78	16	-10.00	10.00
	F	240	230	-4.17	26.67	26	-2.50	8.85
	G	360	390	8.33	40.00	39	-2.50	10.00
	H	500	570	14.00	55.56	51	-8.20	11.18
Mean				5.30			8.87	8.88

Table S5.4: Nominal and measured magnesium and calcium concentrations (mg/L) for all chronic Mg tests. NC = Nourlangie Creek, SB = Sandy Billabong, MC = Magela Creek, GC = Gulungul Creek.

		Magnesium (mg/L)				Calcium (mg/L)				
Test/Site	**Treatment**	**Nominal**	**Measured (av. Start/end, SD) [a]**	**% difference Measured/nominal**	**% difference Start/end**	**Nominal**	**Measured (av. Start/end, SD)**	**% difference Measured/nominal**	**% difference Start/end**	**Mg: Ca Ratio**
1. NC	A - Control	0	0.6			0.00	0.1			6.00
	B	50	46	-8.00		5.56	5	-10.00		9.20
	C	100	100	0.00		11.11	10	-10.00		10.00
	D	150	150	0.00		16.67	15	-10.00		10.00
	E	200	200	0.00		22.22	20	-10.00		10.00
	F	250	260	4.00		27.78	27	-2.80		9.63
	G	300	310	3.33		33.33	31	-7.00		10.00
	H	350	350	0.00		38.89	36	-7.43		9.72
2. SB	A - Control	0	1.1 (0.3)		44.44	0.00	0.25 (0.1)		50.00	4.40
	B	25	26 (0.0)	4.00	0.00	2.78	3 (0.0)	8.00	0.00	8.67
	C	50	51 (0.0)	2.00	0.00	5.56	5.7 (0.0)	2.60	0.00	8.95
	D	100	100 (0.0)	0.00	0.00	11.11	12 (0.0)	8.00	0.00	8.33
	E	150	160 (0.0)	6.67	0.00	16.67	17 (0.0)	2.00	0.00	9.41
	F	200	210 (0.0)	5.00	0.00	22.22	22 (0.0)	-1.00	0.00	9.55
	G	250	265 (7.1)	6.00	3.85	27.78	28 (0.0)	0.80	0.00	9.46
	H	300	305 (7.1)	1.67	-3.23	33.33	33.5 (0.7)	0.50	-2.94	9.10

Table S5.4 (continued)…

Test/Site	Treatment	Magnesium (mg/L)				Calcium (mg/L)				Mg: Ca Ratio
		Nominal	Measured (av. Start/end, SD) [a]	% difference Measured/nominal	% difference Start/end	Nominal	Measured (av. Start/end, SD)	% difference Measured/nominal	% difference Start/end	
3. SB	A - Control	0	0.7 (0.0)		0.00	0.00	0.1 (0.0)		0.00	7.00
	B	50	52 (1.4)	4.00	3.92	5.56	5.55 (0.1)	-0.10	1.82	9.37
	C	100	110 (0.0)	10.00	0.00	11.11	11 (0.0)	-1.00	0.00	10.00
	D	150	160 (0.0)	6.67	0.00	16.67	16 (0.0)	-4.00	0.00	10.00
	E	200	220 (0.0)	10.00	0.00	22.22	22 (0.0)	-1.00	0.00	10.00
	F	250	275 (7.1)	10.00	3.70	27.78	28 (0.0)	0.80	0.00	9.82
	G	300	325 (7.1)	8.33	3.13	33.33	32.5 (0.7)	-2.50	3.13	10.00
	H	400	420 (14.1)	5.00	4.88	44.44	42.5 (2.1)	-4.38	7.32	9.88
4. MC	A - Control	0	0.55 (0.1)		20.00	0	0.15 (0.1)		-50.00	3.67
	B	25	22.5 (6.4)	-10.00	50.00	2.78	2.65 (0.6)	-4.60	40.91	8.49
	C	50	52.5 (2.1)	5.00	5.88	5.56	5.8 (0.1)	4.40	3.51	9.05
	D	100	105 (7.1)	5.00	10.00	11.11	12 (0.0)	8.00	0.00	8.75
	E	150	160 (0.0)	6.67	0.00	16.67	17 (0.0)	2.00	0.00	9.41
	F	200	220 (0.0)	10.00	0.00	22.22	23 (0.0)	3.50	0.00	9.57
	G	275	290 (0.0)	5.45	0.00	30.56	30 (0.0)	-1.82	0.00	9.67
	H	350	375 (7.1)	7.14	2.70	38.89	39 (0.0)	0.29	0.00	9.62

Table S5.4 (continued)…

Test/Site	Treatment	Magnesium (mg/L)				Calcium (mg/L)				Mg: Ca Ratio
		Nominal	Measured (av. Start/end, SD) [a]	% difference Measured/nominal	% difference Start/end	Nominal	Measured (av. Start/end, SD)	% difference Measured/nominal	% difference Start/end	
5. MC	A - Control	0	0.6 (0.0)		0.00	0.00	0.1 (0.0)		0.00	6.00
	B	50	52 (1.4)	4.00	3.92	5.56	5.6 (0.0)	0.80	0.00	9.29
	C	100	110 (0.0)	10.00	0.00	11.11	11 (0.0)	-1.00	0.00	10.00
	D	150	155 (7.1)	3.33	6.67	16.67	16.5 (0.7)	-1.00	6.25	9.39
	E	200	210 (0.0)	5.00	0.00	22.22	21.5 (0.7)	-3.25	-4.55	9.77
	F	250	270 (14.1)	8.00	7.69	27.78	28 (0.0)	0.80	0.00	9.64
	G	300	330 (14.1)	10.00	6.25	33.33	33.5 (0.7)	0.50	3.03	9.85
	H	400	430 (14.1)	7.50	4.76	44.44	44.5 (0.7)	0.13	2.27	9.66
6. GC	A - Control	0	0.5			0.00	0.1			5.00
	B	25	27	8.00		2.78	2.9	4.40		9.31
	C	50	54	8.00		5.56	5.7	2.60		9.47
	D	75	81	8.00		8.33	8.4	0.80		9.64
	E	100	110	10.00		11.11	11	-1.00		10.00
	F	150	170	13.33		16.67	17	2.00		10.00
	G	200	220	10.00		22.22	22	-1.00		10.00
	H	300	320	6.67		33.33	33	-1.00		9.70
Mean				6.09	5.78			3.30	5.49	9.03

[a] For tests 3 and 4 only start of test concentrations were measured.

Table S5.5: Measured magnesium and calcium concentrations (mg/L) for one chronic Mg test, showing minimal differences between averaged total and dissolved concentrations from samples taken at the start and end of the test. All dissolved samples were filtered to 0.45 µm.

Test 1643M – Sandy Billabong	Average magnesium concentration (mg/L)			Average calcium concentration (mg/L)		
	Nominal	Measured Total	Measured Dissolved	Nominal	Measured Total	Measured Dissolved
A - Control	0	1.25	1.1	0.00	0.3	0.25
B	25	27	26	2.78	3.1	3
C	50	51	51	5.56	5.75	5.7
D	100	100	100	11.11	11.5	12
E	150	160	160	16.67	17	17
F	200	210	210	22.22	22	22
G	250	265	265	27.78	28	28
H	300	310	305	33.33	34	33.5

CHAPTER 6: Acute sensitivity of the glochidia of two tropical Australian freshwater mussel species to copper and uranium, and an investigation of interspecies variability in sensitivity to copper.

Abstract

In many aquatic environments, freshwater mussels play important roles both ecologically and culturally, and knowledge of their sensitivity to contaminants is crucial for their protection. Metal contaminants released in surface waters from anthropogenic activities can elevate natural background concentrations, increasing risk to aquatic organisms from their toxic effects. Limited toxicity data are available for metals such as copper or uranium for the early life stages of tropical freshwater mussel species. In addition, knowledge of inter- and intra-species variability in sensitivity to these contaminants is important where data are used for environmental management. The present study evaluated the toxicity of the metals copper and uranium to two tropical freshwater mussel species, *Velesunio angasi* and *Velesunio* sp., to assess sensitivity and inter- and intra-species variability. Acute exposures to larval glochidia were assessed based on 24-h survival. Median lethal (LC50) toxicity estimates for eight copper tests ranged from 5.2 to 8.4 µg/L, while LC50s for six uranium toxicity tests ranged from 227 to 375 µg/L. The results indicated that *Velesunio* spp. were highly sensitive to copper, and similarly sensitive when compared to values reported for other highly sensitive temperate Australian mussel species. No freshwater mussel data was available for uranium, but when compared to other tropical freshwater species tested in waters of similar physico-chemical composition, the results indicated that *Velesunio* spp. were moderately sensitive to uranium. Inter- and intra- species variability in sensitivity to copper was low, and low intra-species variability in sensitivity was indicated for *V. angasi* exposed to uranium. Further data is required for uranium to assess inter-species variability. These results contribute to existing tropical freshwater toxicity data for uranium and copper, and uranium data may be useful for derivation of water quality guidelines for uranium.

6.1 Introduction

Elevated metal concentrations in surface waters occurring through anthropogenic activities can increase the risk of toxic effects on freshwater biota. Exposure to anthropogenic contamination has been recognised as one of the major contributors in the global decline of many freshwater mussel species, with the early life stages considered to be particularly sensitive (Augspurger *et al.* 2007; Lopes-Lima *et al*. 2017). Much ecotoxicological research on freshwater mussels has focused on temperate species from North America, which is home to the world's richest freshwater mussel fauna (Haag and Williams 2014). In contrast, relatively little ecotoxicological research has been undertaken on species from tropical climates.

Copper (Cu) is a common metal contaminant in freshwater systems, and is frequently used as a reference toxicant in ecotoxicological studies. Although it is an essential micronutrient in low concentrations, Cu is known to be highly toxic to invertebrates (USEPA 2007). The early life stages of freshwater mussels have been reported as being particularly sensitive to Cu (Augspurger et. al 2007, March *et al*. 2007). While Cu toxicity data are available for temperate freshwater mussel species from the Northern Hemisphere (Jacobson *et al*. 1997; Wang *et al*. (2007a), New Zealand (Clearwater *et al.* 2014), and Australia (Markich 2017), the effect of Cu on the early life stages of tropical freshwater mussels has not been reported. Copper was selected as a reference toxicant in this study because it occurs universally in contaminated aquatic environments, and is the most commonly tested metal for freshwater mussels for which data are available to compare inter- and intra- species sensitivity (USEPA 2007).

Uranium (U) is a ubiquitous metal in the environment at varying concentrations, depending on its proximity to natural sources such as parent materials (i.e. rocks) or anthropogenic sources such as U mining. For example, in areas with a low geological presence of uraniferous rocks, background U concentrations in freshwaters are typically <0.05 µg/L, but can increase to >100 µg/L in areas with high uraniferous geology (CCME 2011). In addition, concentrations >3000 µg/L have been reported in surface waters associated with acid mine water seepage (Neves *et al*. 2005). Discharges of waste waters from U mining and milling operations can negatively impact the surrounding environment, and efforts to understand the toxic effects of U on freshwater biota have been ongoing for at least the past 30 years (e.g. Hyne *et al.* 1992; Holdway 1992; Semaan *et al*. 2001; Antunes *et al*. 2007; Liber *et al*. 2011; Bergmann *et al.* 2018; Reis *et al*. 2018). Although U can induce

both radiological and chemical modes of toxic action, the extremely long decay half-life of the dominant isotope, ^{238}U (4.5 billion years), decreases the risk of radiological toxicity in comparison to chemical toxicity (Sheppard *et al*. 2005; Mathews *et al*. 2009). In aquatic systems, the main toxic species of U are the free (UO_2^{2+}) and hydrated (UO_2OH^+) uranyl ions, and toxicity varies with different water chemistry such as pH, hardness, alkalinity, and dissolved organic carbon (Markich 2002).

In the monsoonal tropics of the Northern Territory (NT), Australia, U is a contaminant of potential ecotoxicological concern due to past and present mining activities (Supervising Scientist 2018b), and as such has been used as a reference toxicant for local freshwater species as part of a long-term monitoring, assessment and research program to ensure environmental protection (Hogan *et al.* 2010). Many ecotoxicological studies using local freshwater species have been carried out to assess the effects of U (see van Dam *et al*., 2017), and toxicity modifying factors such as water hardness (Riethmuller *et al.* 2000; Markich 2013), alkalinity (Riethmuller *et al.* 2000), pH (Franklin *et al.* 2000), and dissolved organic carbon (Trenfield *et al*. 2011b, Trenfield *et al.* 2012; van Dam *et al* 2012), which may influence U bioavailability and toxicity in the extremely soft receiving waters surrounding the mine.

While previous research has assessed the effects of U on a local tropical freshwater mussel species, *Velesunio angasi,* in its adult life stage (Markich *et al.* 2000; Markich 2003), toxicity data are lacking for *V. angasi* and a related species, *Velesunio* sp. in their sensitive early life stages. Inter- and intra- species variability in sensitivity has been determined to be low for the glochidia of *Velesunio* spp. in previous research with ammonia (Kleinhenz *et al.* 2018) and magnesium (Kleinhenz *et al*. 2019b), but is unknown for other contaminants. Knowledge of inter- and intra- species variations in sensitivity between closely related test species is important when using sensitivity data for environmental management purposes, because differences in sensitivity will influence which species are more appropriate to use in toxicity assessments.

The aims of the present study were to assess the acute toxicity of two metal contaminants, Cu and U, to the glochidia of *V. angasi* and *Velesunio* sp., and thus determine the degree of inter- and intra- species variability in sensitivity to both contaminants. The results provide new Cu and U toxicity data for these species that can be used to inform water management and mine-site rehabilitation practices in tropical environments with very soft receiving waters.

6.2 Materials and methods

6.2.1 Test species and field collections

The two related freshwater mussel species used throughout this study, *Velesunio angasi* and the undescribed *Velesunio* sp., belong to the Hyriidae family, and are found throughout tropical northern Australia. Recent genetic analysis (Environmental Research Institute of the Supervising Scientist, Australian Department of the Environment and Energy, Canberra, ACT, Australia, and Griffith University, Brisbane, Qld, Australia, unpublished data) has identified the presence of these two species within the ARR; *V. angasi* typically inhabits lentic water bodies such as billabongs and lakes, while *Velesunio* sp. is found in lotic systems such as streams, creeks and floodplains that cease to flow during the dry season (June – November). For Cu tests, adult mussels were collected between November 2016 and August 2018 from four locations (Sites 2 – 5, Table 6.1), and for U tests, adult mussels were collected between May 2018 and August 2018 from three locations (Sites 1, 2, and 4, Table 6.1) during this study. Mussels were placed in 20-L plastic aerated drums containing a ~15 cm layer of field-collected sand that had been pre-sterilised by heating to 60°C for 48-72 hours, and ~15 L of site water. The sand allowed the mussels to burrow, thus reducing the chance of early glochidia release caused by stress from movement during transport. Mussels were transported to the laboratory by road at ambient temperature (~28 to 32°C) within 4 h of collection.

Table 6.1: Details of each mussel collection site.

Site	**Species**	**Site**	**Catchment region**	**Latitude (S)**	**Longitude (E)**
1	*Velesunio angasi*	Nourlangie Creek	South Alligator River	12° 51’ 22.01’’	132° 46’ 38.42’’
2	*Velesunio angasi*	Sandy Billabong	South Alligator River	12° 54’ 09.38’’	132° 46’ 50.52’’
3	*Velesunio angasi*	Lake Bennett	Adelaide River	12° 57’ 33.25’’	131° 09’ 52.78’’
4	*Velesunio* sp.	Magela Creek	East Alligator River	12° 40’ 58.93’’	132° 56’ 23.30’’
5	*Velesunio* sp.	Gulungul Creek	East Alligator River	12° 39’ 20.86’’	132° 52’ 42.43’’

6.2.2 Glochidia collection and viability testing

Mature glochidia were collected from adult female mussels by placing each individual mussel flat into a polypropylene container (~10 cm diameter x 4 cm depth) and adding a 50/50 mixture of field-collected water and Magela Creek water (~2 cm depth) until the top of each mussel shell was slightly exposed. Glochidia were released from adult females within 12-h, as a stress response. Viability checks of collected mature glochidia were undertaken by exposing a subsample (~100) of glochidia to a concentrated salt (240 g/L NaCl) solution, and assessing the valve-closure response of glochidia both before and after salt exposure. Viability was calculated using the following formula, as recommended in ASTM (2006):

$$\%\ survial\ = 100\ \times \frac{(\#\ closed\ after\ NaCl\ added - \#\ closed\ before\ NaCl\ added)}{(Total\ \#\ open\ \&\ closed\ after\ NaCl\ added)}$$

Achievement of ≥80% viability in each subsample indicated that remaining glochidia from that mussel were viable and could be used in toxicity tests. Viable mature glochidia were obtained and pooled from more than one mussel where possible, and acclimated to the test diluent (Magela Creek water) for 3-4 h during a series of 50% water changes.

6.2.3 Test diluent

The test diluent used throughout this study, Magela Creek water, was sourced from a permanent billabong of Magela Creek (Bowerbird Billabong, latitude 12°40’28’’S, longitude 132°55’52’’E), situated upstream of the Ranger uranium mine site. The Magela Creek water is representative of the extremely soft-waters of northern Australia, being slightly acidic (pH 5.5 – 6.5), with low electrical conductivity (5 – 20 µS/cm), low ionic strength (hardness 3 – 6 mg/L as $CaCO_3$), and low alkalinity (5 – 10 mg/L as $CaCO_3$, van Dam *et al.* 2010). Water was collected approximately monthly, by pumping into 20-L acid washed plastic containers using a battery-operated inline pump. Water was transported back to the laboratory by road at ambient temperature (28 - 32°C) within 4 h of collection, then filtered using a 3 µm Sartopure PP2 MidiCap filter (Sartorius Stedim). Filtered and un-filtered (Minisart RC 25, 0.45 µm filter, Sartorius Stedim) subsamples of each batch of Magela Creek water were taken for analyses of alkalinity (APHA 2005, method 2320B; Envirolab) within 48 h of collection, and total and dissolved organic carbon (Shimadzu TOC-V CSH), and the remaining waters were refrigerated at 4°C until use in toxicity tests. Average physico-chemical properties of the Magela Creek water used throughout this study are shown in Table 6.2.

Table 6.2: Mean physico-chemical composition of 0.45 µm filtered Magela Creek water (MCW) used in Cu and U toxicity tests. Values represent the mean measurements ± standard deviation of control samples taken at the start of each test.

Physicochemical variable	Copper tests (*n* = 8)	Uranium tests (*n* = 6)	
pH	6.14 ± 0.12	6.09 ± 0.04	
Conductivity (µS/cm)	17.1 ± 1.7	16.0 ± 2.1	
Dissolved oxygen (%)	103.5 ± 4.3	107.3 ± 4.6	
Temperature (°C)	27.3 ± 0.2	27.1 ± 0.3	
Alkalinity (mg/L as $CaCO_3$) [a]	3.5 ± 2.3	<1.7 ± 1.0	
Hardness (mg/L as $CaCO_3$)	3.3 ± 0.7	3.2 ± 0.5	
DOC (mg/L) [a]	2.5 ± 0.7	1.9 ± 0.4	
Analyte	**0.45 filtered MCW**		**Detection limit**
Al (µg/L)	16.0 ± 5.7	13.7 ± 2.9	0.1
Cd (µg/L)	<0.02	<0.02	0.02
Co (µg/L)	0.06 ± 0.01	<0.1	0.01
Cr (µg/L)	0.1 ± 0.1	<0.1	0.1
Cu (µg/L)	0.12 ± 0.03	0.16 ± 0.07	0.01
Fe (µg/L)	65.6 ± 15.9	61.2 ± 5.6	1
Mn (µg/L)	2.03 ± 0.74	1.65 ± 0.25	0.01
Ni (µg/L)	0.21 ± 0.09	0.32 ± 0.08	0.01
Pb (µg/L)	0.07 ± 0.04	0.05 ± 0.01	0.01
Se (µg/L)	<0.2	<0.2	0.2
U (µg/L)	0.098 ± 0.239	0.031 ± 0.017	0.001
Zn (µg/L)	1.0 ± 0.5	1.0 ± 0.6	0.1
Ca (mg/L)	0.1 ± 0.1	0.1 ± 0.1	0.1
Mg (mg/L)	0.7 ± 0.2	0.7 ± 0.1	0.1
Na (mg/ L)	1.8 ± 0.7	1.7 ± 0.3	0.1
SO_4 (mg/L) [b]	93.7 ± 3.6	90.0 ± 3.6	0.5

[a] Measurements for alkalinity and dissolved organic carbon were taken from subsamples of each batch of Magela Creek water.

[b] Higher concentrations of SO_4 were due to the addition of HEPES buffer. Background SO_4 in MCW is ~ 0.3 mg/L.

6.2.4 Preparation of test solutions

Before use in experiments, all plastic and glassware was cleaned by soaking in a 5% (v/v) nitric acid (HNO_3, RCI Labscan Ltd.) bath for 24 h, triple rinsing in deionised water (Elix, Millipore), and washing and rinsing in a laboratory dishwasher (Miele) using phosphate free detergent (Dr. Weigert, Neodisher® Laboclean) plumbed with deionised water.

For Cu tests, a 5 mg/L stock solution was prepared by dissolving analytical grade copper sulfate ($CuSO_4 \cdot 5H_2O$ – AnalaR, CAS number: 7758-98-7) in high-purity water (18 MΩ/cm, Milli-Q; Millipore). For U tests, a 50 mg/L uranium stock solution was prepared in the same way using analytical grade uranyl sulfate ($UO_2SO_4.3H_2O$, Ajax Chemicals, CAS number: 1314-64-3). The stock solutions were added to Magela Creek water to prepare nominal test solutions ranging from 5 – 800 µg/L for U tests, and 1 – 20 µg/L for Cu tests. The HEPES buffer (1 mM, N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid, Sigma-Aldrich) was added to each test solution to control pH within ± 0.3 units of the target test pH of 6.0. The use of HEPES was shown in previous research with ammonia to assist in controlling pH fluctuations and was included in the current study for this reason, as Cu and U toxicity can be influenced by pH. The initial pH of test solutions was adjusted if necessary, until the environmentally relevant target pH of 6.0 ± 0.1 unit was reached, using 5% NaOH or 5% H_2SO_4 (Kleinhenz et al. 2018).

6.2.5 Toxicity testing

The acute toxicity of Cu and U to *V. angasi* and *Velesunio* sp. glochidia was assessed in 24-h toxicity tests using a recently optimised test protocol (Kleinhenz *et al.* 2018), which was adapted from existing standard methods (ASTM 2006). Eight tests were conducted with Cu, and six tests were conducted with U, using glochidia that had been newly-released from adult females (within 24 h). Glochidia were selected individually from the pooled acclimated subsample (refer to section 6.2.2) under a stereo microscope (Leica MZ8) using a 1 mm glass pipette, and transferred directly into the test petri dishes containing 30 mL of test solution acclimated to the test temperature (27.5 °C). Glochidia were selected if they had visible adductor muscles, well-developed teeth, open and spontaneously closing valves, and translucent appearance. Three replicates containing 10 glochidia in each replicate were used per test concentration. Petri dishes were arranged randomly and placed in a constant temperature incubator (Labec) set to 27.5 °C for 24 h. At the end of the test, the survival

endpoint was measured according to the viability testing method described in ASTM (2006), with glochidia counted as dead (non-viable) if they did not respond to the salt (NaCl) treatment. Further details of the acute toxicity test method are detailed in Kleinhenz *et al.* (2018) and the method is summarised in Table 6.3.

Table 6.3: Details of the 24-h acute test protocol used for Cu and U toxicity testing.

Test criteria	**Acute toxicity test**
Test organism species	*Velesunio* spp.
Life stage	Glochidia <24 h old post-release from adult female
Toxicity test type	Static renewal
Test duration	24 h
Test vessels	9 cm plastic petri dishes with lids
Test solution volume	30 mL
Dilution water	Natural Magela Creek Water
# of test organisms per vessel	10
# of replicates per concentration	3
Test solution renewal	-
Feeding	-
Aeration	-
Assessment endpoint	Survival as measured by valve closure response after exposure to NaCl solution
Test acceptability	Survival >90% in each control treatment
Temperature	27.5°C ± 1°C
pH	6.0 ± 0.3
pH control	1 mM HEPES buffer adjusted to pH 6.0
Dissolved oxygen	80-120% saturation
Photoperiod	12:12 (light: dark)

6.2.6 Quality Control

Water quality parameters (electrical conductivity, pH, and dissolved oxygen) were measured for subsamples of test water from each treatment at test commencement, and for pooled subsamples from each treatment at the end of each test. Measurements were taken using a WTW Multi 340i meter, and specific probes (pH: Sentix 41, electrical conductivity: Orion 013005MD, dissolved oxygen: CellOx 325). The incubator temperature was monitored every 5 min throughout each test using remote logging software (Testo Saveris 2).

At the start of each toxicity test, water chemistry samples of Milli-Q blanks, procedural Milli-Q blanks and control water samples from each test were analysed for any potential confounding contamination. Samples were analysed for a standard suite of metals and major ions (Al, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Na, Ni, Pb, Se, SO_4, U and Zn) using inductively coupled plasma–atomic emission spectroscopy and inductively coupled plasma–mass spectrometry. To determine measured U or Cu concentrations, subsamples were analysed from each treatment at the start of each test. For four of the six U tests, subsamples from the end of each test were also analysed to measure the extent of U loss from the test system. Samples were filtered to 0.45 μm (Minisart RC25, Sartorius Stedim), acidified (1% v/v HNO3, Sigma-Aldrich), and analysed at a NATA accredited laboratory (Envirolab, Chatswood NSW) using inductively coupled plasma–mass spectrometry and inductively coupled plasma–atomic emission spectroscopy.

Tests were deemed valid where there was ≥90% survival of control organisms at 24 h, electrical conductivity (EC) at the end of the test remained within 10% of the values measured at the start of the test, average changes in pH within a treatment did not exceed ± 0.3 units, dissolved oxygen concentration remained above 80%, and the incubator temperature remained within 1°C of the target temperature.

6.2.7 Statistical analysis

At the end of each toxicity test, survival data for each treatment were transformed to percentage of the control response where survival was <100%. Measurements from the start of each test were used for Cu tests, and average measurements from the start and end of each test were used for four of the U tests. Calculations of the average loss from the four measured tests were consistent (see Results and Discussion), and were therefore used to estimate end concentrations for the remaining two tests to provide more accurate toxicity estimates. Concentration-response modelling was undertaken using non-linear regression (3-parameter log-logistic) to estimate median lethal concentrations (LC50s) and their 95% confidence limits (CETIS™ v1.9.0.9, Tidepool Scientific, LLC).

6.3 Results and discussion

6.3.1 Quality control

All toxicity tests met test acceptability criteria (± standard deviation) for control performance (Table 6.4); with control survival averaging 97.5% (range 96.7 – 100%) for Cu tests, and 98.9% (range 96.7 – 100%) for U tests. Key mean physicochemical variables (electrical conductivity, pH, dissolved oxygen concentration, and temperature) of control and treatment water samples taken at 0 and 24 h were maintained within the test acceptability criteria for all tests (Table 6.4). No metal or major ion contamination was measured in controls, blanks, or procedural blanks from any of the tests, and the composition (alkalinity, hardness, and dissolved organic carbon) of the Magela Creek water and measured chemical concentrations of the control treatments were consistent with normal Magela Creek water composition, except for higher concentrations of SO_4 due to the addition of HEPES buffer (Table 6.2, and section 6.2.3). Previous research has shown that sulfate is not toxic at these concentrations to other freshwater species in Magela Creek water (van Dam *et al*. 2010).

Measured Cu concentrations were lower than nominal concentrations for most treatments, with a net average difference of 12.7% for all tests (*n = 8*). Loss of Cu between the start and end of tests could not be calculated because end of test concentrations were not measured. Some adsorption of U to the test container surfaces was anticipated, based on previous studies with the freshwater snail, *Amerianna cumingi* (Hogan *et al*. 2010; Mooney *et al*. 2016). In the present study, the average loss of U between nominal and starting concentrations was 15% (range 10 – 20%), and the average loss between the start and end of tests averaged 28% (range 24 - 31%). This may be attributable to binding of U to the test apparatus (plastic test solution bottles and Petri dishes), and is in accordance with the findings of Hogan *et al.* (2010) in a 24-h uptake and binding experiment with snails. They determined a 25% overall loss of U from the test solution, with the majority bound to test apparatus rather than to the test organism (4 – 6%) or food (2 – 3%), as determined through tissue analysis.

Table 6.4: Mean physico-chemical variables of test solutions across all definitive 24-h acute U and Cu tests with *Velesunio* spp. glochidia (± standard deviation), and acute toxicity values for each toxicity test, expressed as LC50 (95% CI). NC = Nourlangie Creek, SB = Sandy Billabong, MC = Magela Creek, LB – Lake Bennett, GC = Gulungul Creek.

Test	Date	Species	Site	Control survival (%)	EC (µS/cm) [a]	pH (Units) [a]	Dissolved Oxygen (%) [a]	Temp. (°C) [b]	DOC mg/L	# treatments
Copper tests										
1	25/11/16	*Velesunio angasi*	SB	96.7	17.0 ± 0.0	6.33 ± 0.3 [c]	105.8 ± 2.7	27.5	3.3	7
2	26/11/16	*Velesunio angasi*	SB	100	17.6 ± 0.5	6.24 ± 0.3 [c]	97.6 ± 4.0	27.4	3.3	7
3	27/05/17	*Velesunio angasi*	SB	100	15.2 ± 0.8	6.09 ± 0.1	99.6 ± 0.7	27.7	1.6	7
4	11/01/17	*Velesunio angasi*	LB	93.3	18.1 ± 0.8	6.02 ± 0.1	101.6 ± 0.2	27.1	3.1	8
5	22/08/18	*Velesunio angasi*	LB	100	17.7 ± 1.4	6.14 ± 0.2	109.4 ± 7.1	27.1	1.7	8
6	26/05/18	*Velesunio* sp.	MC	100	15.4 ± 0.5	6.06 ± 0.1	104.1 ± 5.2	27.2	2.3	8
7	27/05/18	*Velesunio* sp.	MC	100	15.2 ± 0.8	6.08 ± 0.1	105.8 ± 4.4	27.1	2.3	8
8	20/04/18	*Velesunio* sp.	GC	90	18.3 ± 0.6	6.09 ± 0.1	102.5 ± 6.6	27.1	2.0	8
		Mean ± SD		*97.5 ± 3.9*	*16.8 ± 1.3*	*6.13 ± 0.1*	*103.3 ± 3.8*	*27.3 ± 0.2*	*2.4 ± 0.7*	
Uranium tests										
1	5/05/2018	*Velesunio angasi*	NC	100	13.1 ± 1.5	6.04 ± 0.05	101.1 ± 7.0	26.6	2.0	8
2	2/06/2018	*Velesunio angasi*	SB	100	15.5 ± 0.5	6.04 ± 0.03	107.1 ± 8.3	27.1	2.3	8
3	3/06/2018	*Velesunio angasi*	SB	96.7	15.8 ± 0.5	6.04 ± 0.06	107.3 ± 8.6	27.0	2.3	8
4	25/07/2018	*Velesunio angasi*	SB	100	16.4 ± 0.2	6.10 ± 0.07	111.7 ± 7.0	27.4	1.4	8
5	26/07/2018	*Velesunio angasi*	SB	96.7	16.4 ± 0.4	6.09 ± 0.09	109.1 ± 8.8	27.1	1.4	8
6	27/05/2018	*Velesunio* sp.	MC	100	21.1 ± 1.3	6.07 ± 0.07	109.9 ± 4.0	27.1	2.3	8
		Mean ± SD		*98.9 ± 1.7*	*16.4 ± 2.6*	*6.06 ± 0.03*	*107.7 ± 3.7*	*27.1 ± 0.3*	*1.9 ± 0.4*	

[a] Values represent measurements of 3 pooled replicate samples at 0 h and 24 h from all treatments for each acute test.
[b] Incubator temperature was monitored at 5-min intervals using data loggers (Testo Saveris™). New test waters were acclimated to the test temperature for 1-2 hours prior to test commencement.
[c] Copper tests 1 and 2 did not include HEPES buffer for pH control.

6.3.2 Acute copper toxicity

The results of the toxicity testing demonstrated that *Velesunio* spp. were acutely sensitive to Cu, with 24-h LC50 estimates for all eight acute tests being < 10 µg/L. The individual LC50s for the eight acute Cu toxicity tests ranged from 5.2 to 8.4 µg/L, varying by 1.6-fold, and the geometric mean for all tests was 6.6 µg/L (Figures 6.1 – 6.3, Table 6.5). The geometric mean LC50s for mussels from each site were similar, being 5.6 µg/L for Magela Creek *Velesunio* sp., 6.4 µg/L for Lake Bennett *V. angasi*, and 7.5 µg/L for Sandy Billabong *V. angasi.*

Reasons for Cu sensitivity and its mechanisms of toxic action in freshwater mussels are largely unknown (Jorge *et al*. 2017), but Cu toxicity to aquatic organisms has been demonstrated to be heavily influenced by the chemical composition of the water, in particular the major cations magnesium, calcium, and sodium, and dissolved organic carbon content (Grosell and Wood 2002; USEPA 2007; Gillis *et al.* 2008). Jacobson *et al*. (1997) found that the sensitivity to Cu of the glochidial stages of five temperate unionid mussel species varied with species, exposure length, temperature, and water hardness, with median lethal concentrations ranging from 26 to 48 µg Cu/L for glochidia of *Lampsilis fasciola* to 46 to 347 µg Cu/L for glochidia of *Pyganodon grandis.*

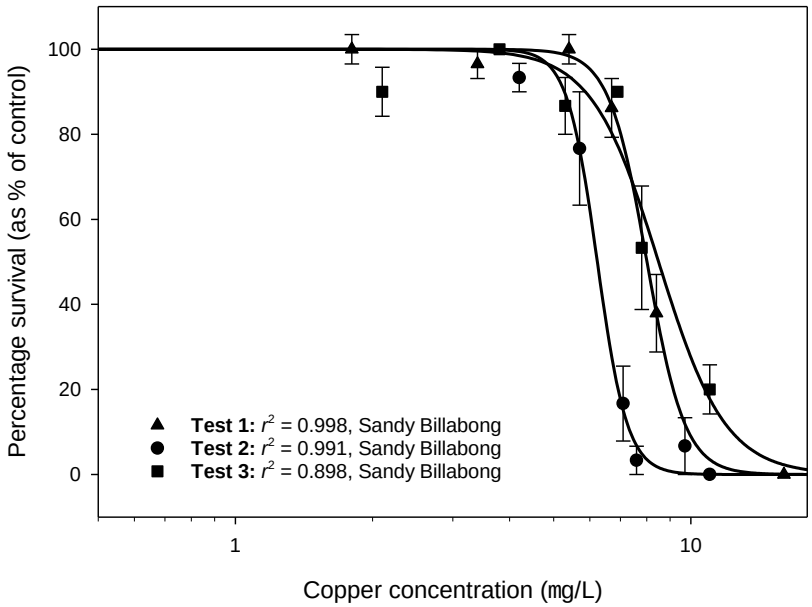

Figure 6.1: Concentration-response relationships for acute 24-h Cu exposures to *Velesunio angasi* from Sandy Billabong. Data points represent the mean ± standard error of 3 replicates. The 95% confidence intervals have been removed for clarity. Toxicity estimates were determined using 3-parameter logistic models.

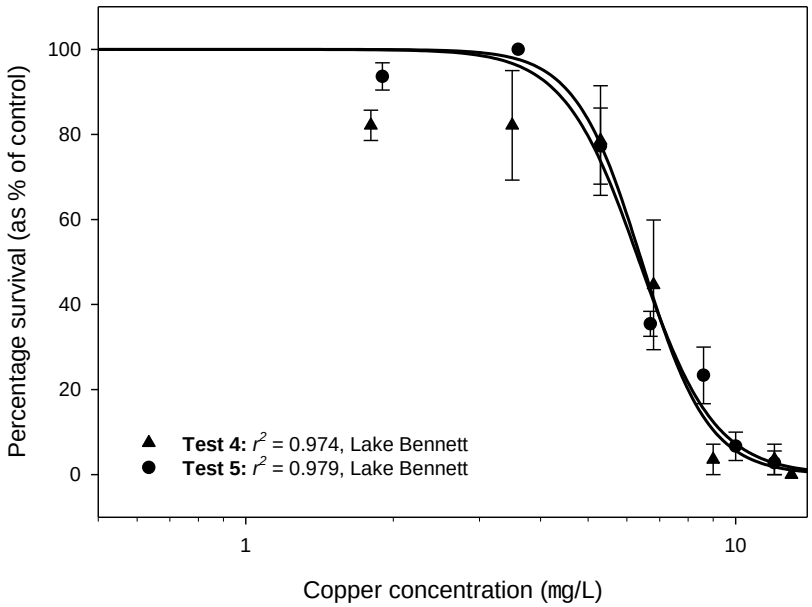

Figure 6.2: Concentration-response relationships for acute 24-h Cu exposures to *Velesunio angasi* from Lake Bennett. Data points represent the mean ± standard error of 3 replicates. The 95% confidence intervals have been removed for clarity. Toxicity estimates were determined using 3-parameter logistic models.

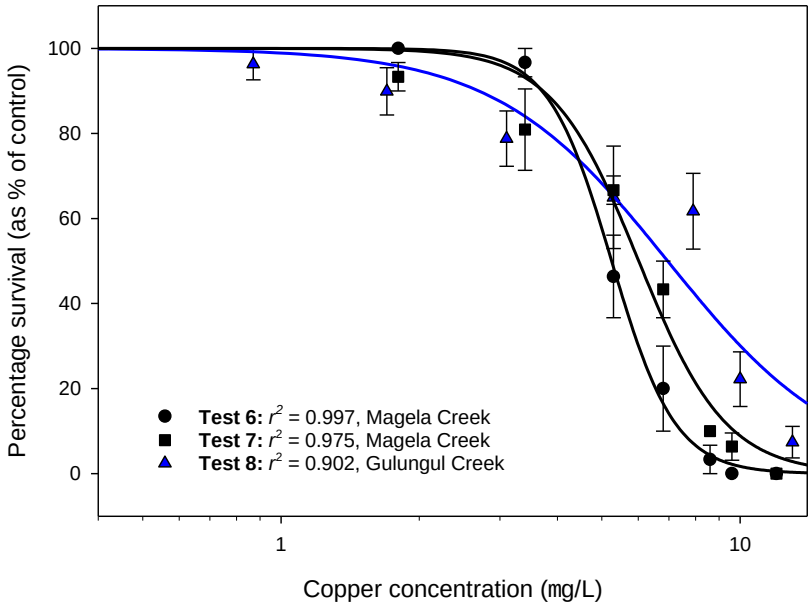

Figure 6.3: Concentration-response relationships for acute 24-h Cu exposures to *Velesunio* sp. from Magela Creek and Gulungul Creek. Data points represent the mean ± standard error of 3 replicates. The 95% confidence intervals have been removed for clarity. Toxicity estimates were determined using 3-parameter logistic models.

Table 6.5: Summary of the Cu and U toxicity estimates (95% CI) for acute 24-h exposures with *Velesunio angasi* (Sandy Billabong, Nourlangie Creek, and Lake Bennett) and *Velesunio* sp. (Magela Creek and Gulungul Creek) [a]

Test	Species	Site	LC50 (µg/L)	Geometric mean (µg/L)	% CV [b]	Mean toxicity value ratio [c]
Copper tests						
1	*Velesunio angasi*	Sandy Billabong	8.0 (7.9 – 8.2)			
2	*Velesunio angasi*	Sandy Billabong	6.2 (6.0 – 6.5)			
3	*Velesunio angasi*	Sandy Billabong	8.4 (7.2 – 9.7)	7.5	15.6	1.4
4	*Velesunio angasi*	Lake Bennett	6.4 (5.7 – 7.0)			
5	*Velesunio angasi*	Lake Bennett	6.4 (5.8 – 6.9)	6.4	0.3	1.0
6	*Velesunio* sp.	Magela Creek	5.2 (5.1 – 5.4)			
7	*Velesunio* sp.	Magela Creek	6.0 (5.4 – 6.6)	5.6	9.5	1.1
8	*Velesunio* sp.	Gulungul Creek	7.0 (5.1 – 9.2)	-	-	-
			All tests	*6.6*	*15.7*	*1.6*
Uranium tests						
1	*Velesunio angasi*	Nourlangie Creek	227 (193 – 265) [d]	-	-	-
2	*Velesunio angasi*	Sandy Billabong	375 (315 – 421)			
3	*Velesunio angasi*	Sandy Billabong	303 (263 – 344)			
4	*Velesunio angasi*	Sandy Billabong	312 (286 – 339)			
5	*Velesunio angasi*	Sandy Billabong	240 (207 – 280)	304	18.2	1.6
6	*Velesunio* sp.	Magela Creek	323 (259 – 395) [d]	-	-	-
			All tests	*292*	*18.5*	*1.6*

[a] The % coefficient of variation and mean toxicity value ratio of the median lethal concentrations (LC50s) for each site are shown as a measure of variability between species and sites.
[b] % CV (coefficient of variation) = standard deviation/mean of LC50 values.
[c] Mean toxicity value ratio = maximum LC50/minimum LC50 values.
[d] Uranium tests 1 and 6 end-of-test concentrations were not measured, but were adjusted by average loss from tests 2 to 5 (28%).

When compared with other freshwater mussels, *Velesunio* spp. were more sensitive to Cu than nine north American temperate species, which had LC50s ranging from 10 to >100 µg/L (Wang *et al*. 2007a). However, these differences may be largely water quality related, as the north American species were exposed to Cu in reconstituted ASTM hard water with a measured hardness of 177 mg/L as $CaCO_3$, a pH of 8.4 and alkalinity of 121 mg/L as $CaCO_3$, in which Cu is likely to have been less bioavailable. Gillis *et al.* (2008) reported LC50s for nine temperate Canadian species, two of which were comparable to *Velesunio* spp. (6.9 and 7.4 µg/L), with the remaining seven being less sensitive than *Velesunio* spp., ranging from 13 to 36 µg/L. *Velesunio* spp. were slightly less sensitive than the temperate New Zealand freshwater mussel species, *Echyridella menziesii* (Hyriidae family; 24-h LC50s ranging from 2.9 to 4.2 µg/L), tested in waters of low hardness (30 mg/L as $CaCO_3$) and low DOC (2.0 – 2.9 mg/L; Clearwater *et al*. 2014). Similar sensitivity was found between *Velesunio* spp. and six temperate Australian Hyriidae species tested in waters of low hardness (42 mg/L as $CaCO_3$) and very low DOC (< 0.1 mg/L), with 24-h LC50s ranging from 6.1 to 10 µg/L (Markich 2017).

6.3.3 Acute uranium toxicity

The individual 24-h LC50s for the six acute U tests with *Velesunio* spp. glochidia (Table 6.5, Figure 6.4) ranged from 227 to 375 µg/L, a 1.7-fold variation. The geometric mean LC50 for all tests was 292 µg/L. No acute U toxicity data were available for freshwater mussel glochidia to compare the sensitivity of *Velesunio* spp. to that of other species. Uranium toxicity has been investigated for a number of other freshwater invertebrate species. For example, LC50s of 8200 µg/L U for the amphipod, *Hyalella azteca*, and 33 500 µg/L U for the midge, *Chironomus dilutus*, were generated by Liber *et al*. (2011) in 96-h water-only tests. However, these tests were conducted in waters of higher pH (8.2), hardness (112 mg/L as $CaCO_3$), and alkalinity (84 mg/L as $CaCO_3$) than the present study. Similarly, higher 96-h LC50s of >262 000 µg /L U for the caddisfly, *Schizopelex festiva*, 142 000 µg/L U for the isopod, *Proasellu* sp., and 24 000 µg/L U for the gastropod, *Theodoxus fluvialtilis*, were generated by Bergmann *et al*. (2018) in waters of higher pH (7.1), and hardness (644 mg/L as $CaCO_3$) than the present study. Generally, the toxicity of U to freshwater organisms has been shown to decrease with increasing pH (Alves *et al*. 2008; Croteau *et al*. 2016), water hardness (Markich 2013; Riethmuller *et al*. 2000) and dissolved organic carbon (Hogan *et al*. 2005; Trenfield *et al*. 2011b), although the direction and degree of these interactions has been shown to be species-specific. Acute toxicity estimates for other and generally more sensitive

freshwater species tested in soft waters are shown in Table 6.6, demonstrating the moderate sensitivity of *Velesunio* spp. to U toxicity.

The effects of different water chemistry on U speciation and toxicity have been demonstrated in other studies, highlighting the importance of site-specific data that consider local conditions. For example, a 17-fold increase in U toxicity to *H. azteca* was demonstrated when the hardness of exposure water was decreased 6-fold (Borgmann *et al*. 2005), while a 4-fold increase in U toxicity was demonstrated for the freshwater macrophyte, *Ceratophyllum demersum*, when hardness was decreased 20-fold (Markich 2013). A 50-fold decrease in hardness only caused a 2-fold increase in U toxicity to the green hydra, *Hydra viridissima*, and no significant increase in U toxicity to the fish, *Mogurnda mogurnda* (Riethmuller *et al*. 2000). Considering that responses to U exposure appear to be species-specific and influenced by water physico-chemistry, further investigations on the toxicity of U under varying physico-chemical conditions may be informative for *Velesunio* species. In particular, DOC is a key variable in the very soft receiving waters of the Magela Creek, and the site-specific water quality limit for U includes a DOC correction (van Dam *et al*. 2017).

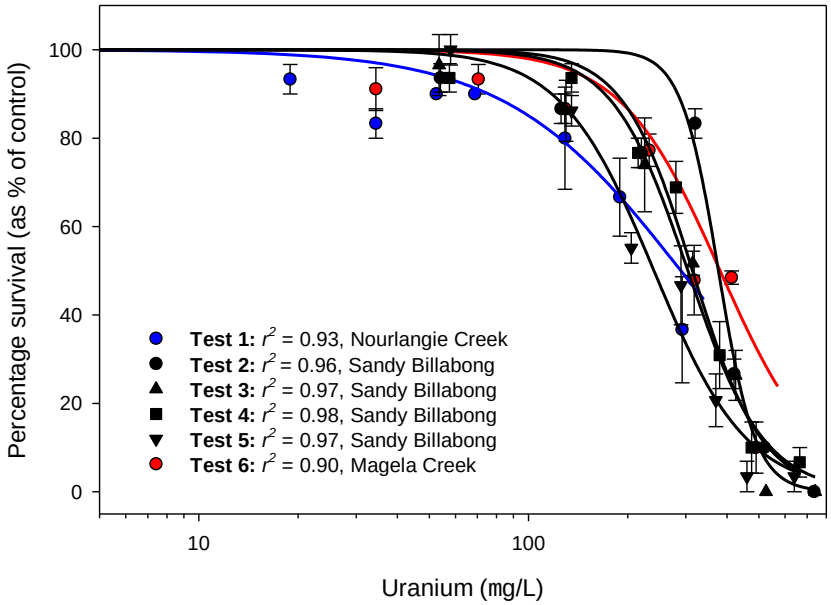

Figure 6.4: Concentration-response relationships for acute 24-h U exposures to *Velesunio angasi* from Nourlangie Creek and Sandy Billabong, and *Velesunio* sp. juveniles from Magela Creek. Data points represent means ± standard error of three replicates per treatment. Toxicity estimates were determined using 3-parameter logistic models.

Table 6.6: Acute U toxicity data for freshwater species in soft waters. Where multiple test data were reported form tests trialling different hardnesses, the lowest values are shown for tests using hardness ≤ 20 (mg/L as $CaCO_3$).

Species	Common name	Test type	Hardness	pH	LC50 (µg/L)	Study
Hyalella azteca	Amphipod	14-d survival	15	6.4 – 7.1	20	Goulet *et al*. (2015)
Hyalella azteca	Amphipod	7-d survival	18	7.4	21	Borgmann *et al*. (2005)
Ceriodaphnia dubia	Water flea	7-d survival	15	6.4 – 7.1	140	Goulet *et al*. (2015)
Pimephales promelas	Fish	4-d survival	15	6.4 – 7.0	160	Goulet *et al*. (2015)
Ceratophyllum demersum	Macrophyte	7-d growth	20	6.2	134	Markich (2013)
Moinodaphnia macleayi	Water flea	48-h survival	~ 1	6.6 – 6.9	160 - 390	Semaan *et al.* (2001)
Velesunio spp.	Freshwater mussel	24-h survival	3.2	6.1	292	This study
Oncorhynchus mykiss	Fish	4-d survival	15	6.4 – 7.0	420	Goulet *et al*. (2015)
Mogurnda mogurnda	Fish	96-h survival	6.6	6.0	1730 - 1965	Riethmuller *et al.* (2000)

6.3.4 Inter- and Intra- species variability in sensitivity

For Cu toxicity tests, comparisons of the LC50s indicated low inter- and intra- species variability in sensitivity, indicating that both species were similarly sensitive to Cu. Inter-species comparisons indicated low variability with a variation of 1.6-fold between all LC50s (Table 6.5). Intra-species variability was similarly low, ranging between no variability to 1.4-fold, and was consistent with intra-species variability reported for temperate mussel species using standardised acute test methods. For example, in the study by Wang *et al.* (2007b), the 24-h LC50s of seven Cu tests with fatmucket (*Lampsilis siliquoidea*) ranged between 29 to 42 µg/L, varying by a factor of 1.4, while the 24-h LC50s of four Cu tests with mucket (*Actinonaias ligamentina*) ranged between 35 to 66 µg/L, varying by a factor of 1.9. In the study by Clearwater *et al.* (2014), the 24-h LC50s of the 3 tests with the temperate New Zealand species, *E. menziesii*, ranged between 2.9 and 4.2 µg/L, varying by a factor of 1.5. Markich (2017) reported 24-h LC50s for six Australian temperate mussel species, ranging from 6.1 to 10.0 µg/L, varying by a factor of 1.6. In the present study, the % CVs of the mean 24-h LC50s within sites (ranging 3.2 – 15.8%) were similar or lower to those for Cu tests with fatmucket (*L. siliquoidea*) of 14% and mucket (*A. ligamentina*) of 25%, which were considered low, indicating good test precision and low intra-species variability (Wang *et al*. 2007b).

Since no clear differences in Cu sensitivity were found between the two *Velesunio* species, the results of the toxicity testing suggested that *Velesunio* sp. and *V. angasi* were similar in sensitivity to Cu, and either species could be used for future toxicity testing. Similar findings were demonstrated with these two species when tested with ammonia (Kleinhenz *et al*. 2018), and magnesium (Kleinhenz *et al*. 2019b) in acute toxicity tests using the same test method. Further acute and chronic toxicity testing with *Velesunio* sp. and *V. angasi*, would be required to determine if the similarity in sensitivity extends to other contaminants. The low variability for sites tested more than once (CV % = 0.3 - 15.6), validated the repeatability of the test method.

Only one U test was undertaken with *Velesunio* sp. from Magela Creek, therefore, inter-species comparisons were difficult to make. However, the LC50 for Magela Creek *Velesunio* sp. (323 µg/L) fell within the range of LC50s for *V. angasi*, indicating similar sensitivity. The LC50s of the four tests with *V. angasi* from Sandy Billabong ranged from 240 to 375 µg/L, a 1.6-fold variation, with a geometric mean of 304 µg/L (Uranium Tests 2 – 5, Table 6.5). demonstrating low intra-species variation in sensitivity. As a further measure of variability,

the coefficient of variation (% CV) of LC50 values for Sandy Billabong tests was 18.2%, indicating low intra-species variability and a robust toxicity test method.

6.4 Conclusion

This study presents the first acute Cu and U toxicity data for the glochidia of two tropical freshwater mussel species; the lentic *V. angasi* and the lotic *Velesunio* sp. The recently refined acute test protocol was successful in deriving high-quality acute toxicity estimates, indicating that both species were moderately sensitive to U and highly sensitive to Cu in comparison with other freshwater taxa. No U toxicity data were available for freshwater mussels to make sensitivity comparisons amongst mussel species. Both species were more sensitive to Cu than many temperate Northern Hemisphere mussel species, and of similar sensitivity to temperate Australian species. The very soft waters of the test diluent likely increased metal toxicity. Intra- and inter-species variability in sensitivity to Cu was not significant between the two species, in agreement with previous research using ammonia and magnesium. Further data is required for *Velesunio* sp. to test this hypothesis for U. These results provide new U and Cu toxicity data for tropical freshwater mussels, which will contribute to the datasets for tropical freshwater species and may be used to inform environmental management in tropical environments.

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CHAPTER 7: Toxicity test optimisation for the assessment of chronic uranium toxicity using juveniles of two tropical Australian freshwater mussels, *Velesunio* sp. and *Velesunio angasi.*

Abstract

Anthropogenic activities such as mining may lead to increased metal concentrations in freshwater ecosystems. Concerns about the toxic effects of uranium (U) mining and milling activities on freshwater biota has led to an increase in U toxicity studies with freshwater biota. However, chronic U toxicity data are lacking for the sensitive early life stages of freshwater mussels. Juvenile mussels are exposed to metals in both the sediment and the water column through their deposit and filter feeding habits, thus exposure to metals may occur through water-borne and dietary exposure routes. Assessing the sensitivity of juvenile mussels to U can be challenging during chronic toxicity tests that require sediment for optimal performance, due to binding of uranium to the sediment components. Seven 14-d chronic U toxicity tests were undertaken with juvenile mussels (*Velesunio* spp.); four tests using sediment pre-spiked with U, and three without pre-spiked sediment. Water chemistry measurements of filtered and total fractions were assessed, along with survival and growth endpoints. Pre-spiking the sediment using different methods did not significantly reduce the loss of U from the water-column to the introduced fine silt, and the spiking methods reduced control growth by 47% to 88% compared to the tests without spiked sediments. Filtered U concentrations were reduced by up to 90%, indicating significant sorption to sediment particles, thereby providing a route for dietary uptake by the juvenile mussels. Median effect (EC50) toxicity estimates for the filtered and total fractions of the three un-spiked sediment tests ranged from 64 to 126 µg/L U (filtered fraction) and 276 to 506 µg/L U (total fraction). The water chemistry results, the sorption of U by the sediment, and the mussels' requirement for sediment to survive the tests suggest that total U concentrations may be more appropriate to use when deriving toxicity estimates for environmental protection of newly transformed juvenile mussels. The results provide valuable information for future chronic toxicity assessments using *Velesunio* spp. juveniles with U and other metals, where sediment is used in the test system.

7.1 Introduction

Metals released into surrounding environments from mining activities can result in concentrations of receiving waters exceeding background levels. In freshwater ecosystems, increased metal concentrations are a potential source of contamination of water and sediment due to their mobility and persistence, and can result in toxic effects on freshwater biota. The bioavailability and toxicity of metals to freshwater organisms is dependent on the chemical form or speciation of the metal, which is influenced by the chemistry of the water and sediment.

Due to increasing concerns about the anthropogenic effects of uranium (U) mining and milling activities to freshwater ecosystems over the past few decades, the toxicity of U to a wide range of freshwater organisms has been studied intensively (Massarin *et al*. 2010). In oxic surface waters, the main physicochemical forms of dissolved U(VI) species include the free (UO_2^{2+}) and the hydrated (UO_2OH^+) uranyl ions, with UO_2^{2+} considered to be the most toxic ion due to its solubility (Markich 2002; Alves *et al*. 2008). Uranium speciation and therefore its bioavailability and toxicity, is dependent on the surface water chemistry. Generally, U toxicity is known to decrease with increasing pH (Alves *et al*. 2008), dissolved organic carbon (DOC; Trenfield *et al*. 2011a, 2011b), hardness (Markich 2013), and alkalinity (Markich *et al*. 1996), although the relationships do not always hold and are species-specific. The mobility of U in sediments also depends on its oxidation state, and the formation and strength of complexes with inorganic ligands (e.g. carbonate or phosphate), and humic substances (e.g. DOC), which limit U uptake and toxicity to biota by reducing concentrations of UO_2^{2+} (Markich 2002).

Acute and chronic toxicity studies have demonstrated the sensitivity of juvenile freshwater mussels to metals such as copper (Wang *et al*. 2007; Giacomin *et al*. 2013; Jorge *et al*. 2013), lead (Wang *et al*. 2010), aluminium (Wang *et al*. 2018a), and mercury (Valenti *et al*. 2005), but toxicity data are lacking for U, in particular chronic data for tropical freshwater mussel species in their early life stages. The limited chronic toxicity data for metals and freshwater mussels, which are based on methods that incorporate silt or sediment in the test system, is partly due to the lack of a standardised test method (Ingersoll *et al*. 2007), although recent studies have adapted water-only test methods to conduct whole-sediment toxicity tests with juvenile mussels (Wang *et al*. 2013).

As benthic sediment-dwellers, juvenile mussels may be exposed to metals in sediment and pore water via pedal-feeding of sorbed particles, and to dissolved metals in the overlying

water via filter-feeding (Cope *et al*. 2008). A significant amount of metal exposure may occur through the mussel diet (Cope *et al*. 2008), especially for juvenile mussels whose initial feeding behaviour is mostly by pedal feeding (Araujo *et al*. 2017). The transition to filter feeding is a gradual process dependent on the development of the filtering organ (ctenidium), and has been shown to be influenced by mussel size rather than age (Schartum *et al*. 2016). However, filter-feeding has been observed in juvenile mussels (*Villosa iris*) up to 14-d old, via an inhalant current created by the cilia on the foot into the pedal gape (Yeager *et al*. 1994). Sediment in the form of fine silt has been shown to be necessary for the culturing of, and chronic toxicity testing for, juvenile mussels (Hudson and Isom 1984). Sediment is used by the mussels as an additional food source to achieve optimal health and survival, and toxicity tests including sediment or silt would therefore provide a more environmentally relevant evaluation of their sensitivity to metals and other contaminants. However, metal binding to sediment particles is a concern when using sediments in chronic toxicity tests, and methods to minimise or account for binding are needed to accurately assess metal contaminants (Simpson *et al*. 2004).

Chronic toxicity data are needed for the derivation and refinement of site-specific water quality guideline values (GVs) in areas of high conservation value, such as in tropical northern Australia in the vicinity of the Ranger uranium mine. Although the mine operates under strict regulation, mine wastewater discharges may contain above average background concentrations of U (Supervising Scientist 2018b), which need to be kept below concentrations that would cause immediate or long-term adverse effects on freshwater species, including mussels. The current site-specific GV for uranium is 2.8 µg/L, and according to biological monitoring data has not been exceeded in creeks or billabongs downstream of the mine during operations. This GV is applicable where DOC concentration is 2 mg/L, but can be adjusted where higher DOC concentrations (> 10 mg/L) occur (Supervising Scientist 2018b). The mussel species found in this region, *Velesunio* spp., are ecologically and culturally important, and have been shown to be highly sensitive to another mine-related contaminant, ammonia (Kleinhenz *et al*. 2018, 2019a). Thus, knowledge of the effects of other mining contaminants is crucial for the protection of mussel species.

The aim of this study was to assess the fate and biological effects of U to freshwater mussels in 14-d toxicity tests, using a test system that contains fine sediment < 63 µm (silt). Different sediment spiking methods were trialled to determine which method, if any, was effective in minimising or accounting for adsorption of dissolved U to the sediment during toxicity tests. The results of this study will be used to refine the chronic toxicity test method

for freshwater mussels for the future assessment of metal toxicity including U. The chronic data will be used for environmental management, such as the refinement of a site-specific water quality GV for U (van Dam *et al*. 2017).

7.2 Materials and methods

7.2.1 Test species and field collections

Adult freshwater mussels of two related species were hand-collected between January 2017 and August 2018 from four different locations throughout this study. *Velesunio* sp., a lotic species, was collected from Magela Creek and Gulungul Creek, and *Velesunio angasi*, a lentic species, was collected from Nourlangie Creek and Sandy Billabong (Table 7.1). Based on previous research with these species indicating similar sensitivity to other toxicants (Kleinhenz *et al*. 2018; 2019a; 2019b; and Chapter 7 of this thesis), the two species were assumed to have similar sensitivity for the purpose of this study.

Mussels were collected into a 20-L plastic aerated drum containing ~15 L of site-collected water, and a 15-cm layer of field-collected sand. The purpose of the sand was to allow mussels to burrow, thus reducing movement and stress during road transport which may result in early release of glochidia. The sand had been pre-treated in the lab by heating to 60°C for 48-72 hours to minimise presence of pathogens. Mussels were transported to the laboratory at ambient temperature (~28 to 32°C) within 4 h of collection, where they were immediately prepared for glochidia release.

Table 7.1: Details of each mussel collection site.

	Species	Site	Catchment region	Latitude (S)	Longitude (E)
1	*Velesunio* sp.	Gulungul Creek	East Alligator River	12° 39’ 20.86’’	132° 52’ 42.43’’
2	*Velesunio* sp.	Magela Creek	East Alligator River	12° 40’ 58.93’’	132° 56’ 23.30’’
3	*Velesunio angasi*	Sandy Billabong	South Alligator River	12° 54’ 09.38’’	132° 46’ 50.52’’
4	*Velesunio angasi*	Nourlangie Creek	South Alligator River	12° 51’ 22.01’’	132° 46’ 38.42’’

7.2.2 Glochidia collection and viability testing

Glochidia were collected from adult female mussels for the host fish attachment according to the method detailed in Kleinhenz *et al.* (2018), which involved placing each mussel into a round plastic container (~10 cm diameter) and adding field-collected water to the top of each mussel shell. Each container was covered and incubated overnight at a constant temperature of ~27.5°C, the low water level inducing the mussels to release any glochidia from their inner gills into the water, usually within 12 h. Mature glochidia released from each adult female were then checked for viability by exposing three subsamples of ~100 glochidia per mussel to a 240 g/L NaCl solution, to assess valve closure response which is crucial for host fish attachment. Batches of glochidia were considered viable if the subsamples achieved a mean viability of ≥80%, calculated using the following formula recommended in ASTM (2006):

$$\%\, survial \; = 100 \times \frac{(\#\, closed\; after\; NaCl\; added - \#\; closed\; before\; NaCl\; added)}{(Total\; \#\; open\; \&\; closed\; after\; NaCl\; added)}$$

Batches of glochidia used in host-fish exposures consisted of viable mature glochidia obtained and pooled from a minimum of three mussels. Glochidia batches were acclimated to filtered Darwin tap water (FTDW) prior to exposure to host fish, by a series of 50% water exchanges over 3 h before host-fish exposures.

7.2.3 Glochidia transformation

Transformation of glochidia into juvenile mussels was undertaken in the laboratory using the host fish, *Mogurnda mogurnda*. Ethical approval to use host fish was obtained from Charles Darwin University's Animal Ethics Committee (Project A15018; Appendix E). This species is a natural fish host for *Velesunio* spp. (Humphrey and Simpson 1985) and was reared in the laboratory from fish that were originally sourced from the Alligator Rivers Region. Each batch of newly-released mature glochidia were exposed to two *M. mogurnda* in 4 L of vigorously aerated Filtered Darwin tap water (FDTW) at a density of ~16 glochidia/mL for 35 min. Exposed fish were transferred to a plastic tub containing FDTW where they were housed for up to 10 d during juvenile mussel transformation and excystment. Juveniles were collected by siphoning them from the bottom of the tub through a 63 µm sieve and placing them into a watch glass with water from the tub. Juveniles were then

acclimated to the test diluent and temperature for 3-4 h before the start of tests. Further detail of the host fish exposure method is detailed in Kleinhenz *et al*. (2019a).

7.2.4 Test diluent

The test diluent, Magela Creek water (MCW), was collected monthly from Bowerbird Billabong (latitude 12°40’28’’S, longitude 132°55’52’’E), a permanent unimpacted water body of Magela Creek situated upstream of the Ranger uranium mine site. These waters are extremely soft, with slightly acidic pH (5.5 – 6.5), low electrical conductivity (5 – 20 µS/cm), low ionic strength (hardness 3 – 6 mg/L as $CaCO_3$), and low alkalinity (5 – 10 mg/L as $CaCO_3$, van Dam *et al*. 2010) being typical for this region. Water was pumped into 20-L acid washed plastic containers using a battery-operated inline pump, and transported back to the laboratory by helicopter and road at ambient temperature (28 - 32°C) within 4 h of collection. Water was filtered in the laboratory using a 3 µm Sartopure PP2 MidiCap filter (Sartorius Stedim), and refrigerated at 4°C until use.

7.2.5 Preparation of test solutions

Plastic and glassware used in all experiments were prepared by soaking in a 5% (v/v) nitric acid (HNO_3) bath for a minimum of 24 h, rinsing in deionised water, then washing in a laboratory dishwasher (Miele) plumbed with deionised water, using phosphate free detergent (Dr. Weigert, Neodisher® Laboclean). A 50 mg/L U stock solution was prepared for toxicity tests by dissolving analytical grade uranyl sulfate ($UO_2SO_4.3H_2O$, Ajax Chemicals) in high-purity water (18 MΩcm, MilliQ; Millipore). Test solutions were prepared by diluting the stock solution with Magela Creek water. Fine sediment (≤63 µm, ~0.14 g/L dry weight; see section 7.2.6) was added to each test solution until a turbidity of 100 NTU was reached (TPS 90-FLT meter, probe 125186). Sediment was pre-spiked with U for tests 1 to 4, but was not pre-spiked for tests 5 to 7. The HEPES buffer (1 mM, N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid, Sigma-Aldrich) was added to each test solution for the purpose of controlling pH within ± 0.3 units of the target test pH (6.0). The pH of the test solutions was adjusted using 5% NaOH or 5% H_2SO_4, to achieve an initial pH of 6.0 ± 0.1.

7.2.6 Water and sediment spiking

A preliminary 14-d test using the chronic toxicity test method (results not shown) and nominal concentrations ranging from 1 to 74 µg/L indicated a loss of up to 78% of U from the nominal concentration of the test water in the 0.45 µm filtered fraction measured at the start of the test. This suggested that dissolved U from the water column may be immediately binding to the added sediment, thus reducing the U concentration in the water column. Subsequently, the aim for further tests was to saturate the U binding sites in the sediment by pre-spiking the sediment with U stock to minimise U loss from the test water. Four toxicity tests (Tests 1 – 4) were carried out using sediments pre-spiked with U. Three tests (Tests 5 – 7) were undertaken using un-spiked sediment and higher test concentration ranges, to account for U loss to the sediment. Throughout the toxicity tests, growth and survival of juveniles were monitored as the endpoints. Concentrations of U were measured in filtered (0.45 µm) and total composite sub-samples taken at the start and end of each test, to determine the proportion of U in the sediment (particulate) and water (dissolved) compartments.

The sediment used in all toxicity tests was collected from the exposed edges of an unimpacted backflow billabong (Sandy Billabong; latitude 12° 54’ 4’’S, longitude 132° 46’ 38’’E), in October 2015. The < 63 µm fraction was obtained by dry sieving, except for Test 1, for which the sediment was wet sieved using MilliQ water. All sediment was heated at 60°C for at least 72 h to minimise pathogens, then stored at -4 °C until use. Each sediment sample contained 0.28 g of dry sediment; the amount needed to achieve 100 NTU in each 2 L treatment volume (0.14 g/L). For tests using spiked sediment, the sediment was spiked in 5 mL plastic vials by adding ~4 mL of that particular treatment concentration in MilliQ water. Controls were treated in the same way as spiked samples, but were overlaid with MilliQ water only. The methods of sediment spiking varied between each test and were adjusted for optimisation purposes. These methods are summarised in Table 7.2.

Table 7.2: Method of sediment spiking for all tests (µg/L U). Spiked samples (~4 mL) were added to each 2 L test concentration in MCW.

Test	Pre-spiked sediment?	Nominal U concentration	Spiked sediment concentration	Details
1	Y	0-184	0-184	Wet sediment was spiked according to each nominal U concentration. Samples were left to settle for 24 h at room temperature. Overlying water (~4 mL) was removed and replaced with 4 mL MilliQ water.
2a	Y	0-1840	0-1840	Dry sediment was spiked according to each nominal U concentration. Spiked samples were put on a rotating mixer for ~24 h at room temperature (~28°C). Samples were washed 3× by centrifuging (7 mins, 2500 rpm) in MilliQ water. Overlying water (~4 mL) was removed and replaced with 4 mL MilliQ water for each wash.
2b	Y	0, 230, 230, 230	0, 460, 920, 1840	3 sediment samples were spiked with U concentrations representing 2×, 4×, and 8× the predicted EC50 value (230 µg/L), to test the effect on water chemistry and juvenile growth and survival. Spiked samples were put on a rotating mixer for ~24 h at room temperature (~28°C). Samples were washed 3× by centrifuging (7 mins, 2500 rpm) in MilliQ water. Overlying water (~4 mL) was removed and replaced with 4 mL MilliQ water for each wash.
2c	N/A	0, 230, 460	No sediment	Three additional test concentrations were prepared without sediment, to test the effect on water chemistry and juvenile growth and survival.
3	Y	0-700	0-700	Dry sediment was spiked according to each nominal U concentration. Spiked samples were put on rotating mixer for ~48 h, then heated for 48 h at 60 °C. Overlying water (~4 mL) was not removed.
4	Y	0-700	0-700	Dry sediment was spiked according to each nominal U concentration. Spiked samples were heated for 24 h at 60 °C. Overlying water (~4 mL) was removed and replaced with 4 mL MCW.
5	N	0-800	-	Dry sediment was added to each test concentration.
6	N	0-800	-	Dry sediment was added to each test concentration.
7	N	0-800	-	Dry sediment was added to each test concentration.

7.2.7 Toxicity testing

Seven 14-d toxicity tests were conducted; four with *Velesunio angasi* from Sandy Billabong and Nourlangie Creek, and three with *Velesunio* sp. from Magela Creek and Gulungul Creek. Based on previous research assessing ammonia and magnesium toxicity to *Velesunio* spp., differences in sensitivity between the two species were considered inconsequential for the purpose of this study (Kleinhenz *et al* 2018, 2019a, 2019b). For all tests, algal food (*Chlorella* sp.) was pipetted into each 175 mL test container at a rate of ~7.5 x 10^4 cells/mL, immediately prior to dispensing each 100 mL test treatment, including during 48-h water changes. Test treatments were warmed to the test temperature prior to test commencement. Juveniles were selected for tests under a stereo-microscope (Leica MZ8), and impartially transferred directly into duplicate test containers for each treatment (10 per replicate) using a 1 mm diameter glass pipette. Juveniles were considered healthy if they were of opaque appearance, internal organs were visible through the partly translucent shell, with visible pedal (foot) movement. Test containers were placed randomly on Perspex trays at 27.5°C in a constant temperature incubator. Heat pads were used during water changes when test containers were removed from the incubator, to ensure that test water temperatures were maintained.

To obtain an average starting measurement, the growth rate endpoint was measured using a Leica M205C microscope fitted with a camera (Leica MC170HD). A subsample of 30 juveniles, which were not used in the exposures, were photographed on day 0. Image analysis software was used to measure the maximum shell length of each mussel, and the average starting length was calculated. On day 8, mussels from each replicate treatment were removed from their test containers, rinsed gently to remove any debris using overlying test water and a plastic pipette, and placed into a 6-well plate (one well per replicate) along with 5 mL of overlying test water, before photographing. For day 14 (end of test) photographs, mussels were prepared the same way, but euthanised in a 6-well plate containing 70% ethanol to minimise movement. Image analysis software was used on the photographs to measure the maximum shell length for each mussel and thus calculate the average daily growth rate for each test concentration on days 8 and 14. Further information on the host fish exposure method and toxicity test protocol is detailed in Kleinhenz *et al*. (2019a), and summarised in Table 7.3.

Table 7.3: Details of the 14-d chronic toxicity test protocol used for U toxicity testing.

Test criteria	Chronic toxicity test
Test organism species	*Velesunio* spp.
Life stage	Juvenile <24 h old post-excystment from host fish
Toxicity test type	Static renewal
Test duration	14 d
Test vessels	6 cm plastic jars (175 mL) with lids
Test solution volume	100 mL
Dilution water	Natural Magela Creek Water
# of test organisms per vessel	10
# of replicates per concentration	2
Test solution renewal	100% every 2 days
Feeding	*Chlorella* algae (~7.0 - 8.0 x 10^4 cells/mL) every 2 days
Aeration	-
Turbidity at start and at water changes	100 NTU using fine sediment ≤63 µm
Assessment endpoint	Growth rate, survival
Test acceptability	Survival >80% in each control treatment
Temperature	27.5°C ± 1°C
pH	6.0 ± 0.3
pH control	1 mM HEPES buffer adjusted to pH 6.0
Dissolved oxygen	80-120% saturation
Photoperiod	12:12 (light: dark)

7.2.8 Quality Control

For each batch of newly collected MCW, subsamples of filtered (Minisart RC 25, 0.45 µm filter, Sartorius Stedim) and non-filtered water were measured for dissolved and total organic carbon (Shimadzu TOC-V CSH), and alkalinity (APHA 2005, method 2320B; Envirolab). For each toxicity test, electrical conductivity, pH, and dissolved oxygen concentrations were measured using a WTW Multi 340i meter and specific probes (pH: Sentix 41, electrical conductivity: Orion 013005MD, dissolved oxygen: CellOx 325). Water quality parameters were measured on subsamples of new and old water during 48-h water changes. For reported parameters, measurements on new and old water from each treatment were averaged, with "new" water referring to fresh test water, and "old" water referring to water mussels had been exposed to for 48 h. Remote data logging software (Testo Saveris™) was used to record and monitor the incubator temperature every 5 minutes, which was set to the test temperature of 27.5 °C.

Samples of Milli-Q blanks, procedural Milli-Q blanks and control water for each test were analysed for a standard suite of metals and major ions (Al, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Na, Ni, Pb, Se, SO4, U and Zn) at a NATA accredited laboratory (Envirolab, Chatswood NSW) using inductively coupled plasma–atomic emission spectroscopy and inductively coupled plasma–mass spectrometry. Filtered (0.45-µm filtered) and unfiltered (total) subsamples were also taken for each treatment at the start and end of each test and analysed for uranium only. The unfiltered (total) subsamples were taken from the new and old waters that had been stirred immediately prior to subsampling to ensure that any settled silt particles were captured in the sample. Samples were filtered to 0.45 µm (Minisart RC25, Sartorius Stedim), acidified (1% v/v HNO3, Sigma-Aldrich), and refrigerated at 4 °C until analysis.

Toxicity tests were considered acceptable if: survival of control organisms was ≥ 80% at the end of each chronic toxicity test; minimum growth of control organisms at the end of chronic tests was between 13–32 µm/day and comparable to the average control growth rate of 21.2 µm/day (± 5.8 standard deviation; n = 18), based on data obtained in Kleinhenz *et al.* (2019a); average changes in pH remained within ± 0.3 units between new and old waters; changes in electrical conductivity (EC) remained within 10% between new and old waters; dissolved oxygen concentration remained ≥ 80%; and temperature remained within 1°C of the target temperature of 27.5 °C throughout each test.

7.2.9 Statistical analysis

Measured concentrations from the start and end of each test were averaged for both the filtered (< 0.45 µm) and total U fractions for use in concentration-response modelling (Tests 2, 4 – 7). Non-linear regressions (3-parameter log-logistic) were used to estimate 10% and 50% effect concentrations (EC10s and EC50s) from chronic toxicity test effect data (expressed as percentage of control growth), along with their 95% confidence intervals, using CETIS™ (v1.9.0.9, Tidepool Scientific, LLC).

7.3 Results

7.3.1 Quality control

The composition of the test diluent, Magela Creek water, was consistent throughout all tests, with measured pH, conductivity, dissolved oxygen, alkalinity, hardness, and DOC concentrations typical of normal Magela Creek water composition. Measured chemical concentrations of the filtered 0.45 µm fraction of control treatments were consistent with those found in normal Magela Creek water, apart from concentrations of SO_4 which measured higher than normal background concentrations of ~0.3 mg/L, due to the addition of HEPES buffer (Table 7.4, and section 7.2.4 *Test diluent*). Measured analytes in the total fraction were higher than the filtered fraction in most cases, particularly for Al, Fe, Pb, and U, and reflected the composition of the sediment added as an additional food source (Table 7.4).

All toxicity tests met test acceptability criteria (± standard deviation) for control survival except for Test 3, which achieved 75% control survival, and Test 2c, which only achieved 45% control survival (Table 7.5). Decreased survival was expected in Test 2c due to the omission of sediment. Control survival averaged 95.7% (range 90 – 100%) for all other tests. Test acceptability criteria for control juvenile growth rate was met for two of the tests using pre-spiked sediments, and all three of the tests using un-spiked sediment. Those that met the criteria ranged from 14.1 to 28.4 µm/day, averaging 21.8 ± 7.2 µm/day, consistent with the average control growth rate (21.2 ± 5.8 µm/day) and minimum growth rate (12.8 µm/day) deemed acceptable for these species in previous research (Kleinhenz *et al*. 2019a).

Water quality parameters for key variables were maintained within the test acceptability criteria for all tests (± standard deviation) as follows: Electrical conductivity averaged 15.7 ± 1.9 µS/cm, pH averaged 6.04 ± 0.03, dissolved oxygen concentration remained ≥ 80%, averaging 109.7 ± 4.3%, and temperature averaged 27.0 ± 0.2 °C (Table 7.5). No metal or major ion contamination was evident in blanks or procedural blanks from any of the tests. Loss of dissolved U to the sediment, food, and test apparatus was expected, based on a preliminary test and the findings of previous studies (Hogan *et al*. 2010; Mooney *et al*. 2016).

Table 7.4: Average physico-chemical composition of 0.45 µm filtered and total fractions of Magela Creek water used in chronic toxicity tests. Values represent the mean measurements ± standard deviation of control subsamples across all tests.

Physicochemical variable			
pH	6.05 ± 0.03		
Conductivity (µS/cm)	15.6 ± 1.9		
Dissolved oxygen (%)	110.0 ± 5.5		
Temperature (°C)	27.0 ± 0.2		
Alkalinity (mg/L as $CaCO_3$) [a]	1.7 ± 1.0		
Hardness (mg/L as $CaCO_3$)	3.2 ± 1.0		
DOC (mg/L) [a]	2.3 ± 0.8		
Analyte	**Filtered fraction (*n* = 7 tests)**	**Total fraction (*n* = 2 tests)**	**Detection limit**
Al (µg/L)	19.7 ± 5.3	567.5 ± 51.2	0.1
Cd (µg/L)	<0.02	<0.03	0.02
Co (µg/L)	0.10 ± 0.03	0.61 ± 0.17	0.01
Cr (µg/L)	<0.1	0.7 ± 0.2	0.1
Cu (µg/L)	0.17 ± 0.05	1.85 ± 0.21	0.01
Fe (µg/L)	74 ± 20	763 ± 152	1
Mn (µg/L)	6.13 ± 1.69	14.00 ± 2.94	0.01
Ni (µg/L)	0.22 ± 0.07	0.55 ± 0.03	0.01
Pb (µg/L)	0.05 ± 0.02	10.01 ± 0.28	0.01
Se (µg/L)	<0.2	<0.2	0.2
U (µg/L)	0.019 ± 0.007	0.365 ± 0.253	0.001
Zn (µg/L)	0.5 ± 0.2	1.8 ± 0.4	0.1
Ca (mg/L)	0.2 ± 0.1	0.3 ± 0.1	0.1
Mg (mg/L)	0.6 ± 0.1	0.9 ± 0.4	0.1
Na (mg/ L)	1.8 ± 0.3	2.0 ± 0.3	0.1
SO_4 (mg/L) [b]	91.4 ± 4.1	95.0 ± 1.8	0.5

[a] Measurements for alkalinity and dissolved organic carbon were taken from subsamples of each batch of Magela Creek water.
[b] Higher concentrations of SO_4 were due to the addition of HEPES buffer. Background SO_4 in MCW is ~ 0.3 mg/L.

Table 7.5: Summary of the chronic 14-d tests with *Velesunio* spp. juveniles showing survival and average control growth for each control treatment, and mean physico-chemical variables of test solutions across all treatments (± standard deviation). Tests 1-4 included U-spiked sediment, and tests 5-7 included un-spiked sediment. SB = Sandy Billabong, MC = Magela Creek, GC = Gulungul Creek, NC = Nourlangie Creek.

Test	Date	Species	Site	Control survival (%)	Mean control growth (µm/day)	Physico-chemical variables			
						Conductivity (µS/cm) [a]	pH (Units)[a]	Dissolved oxygen (%) [a]	Temp. (°C) [b]
U-spiked sediment:									
1	31/10/2017	*Velesunio angasi*	SB	95	14.1	18.3 ± 0.6	6.07 ± 0.08	115.8 ± 7.8	27.1
2a	22/12/2017	*Velesunio* sp.	MC	90	6.6	17.2 ± 0.7	6.00 ± 0.04	107.5 ± 9.0	27.1
2b	22/12/2017	*Velesunio* sp.	MC	90	6.6	16.6 ± 0.5	6.00 ± 0.03	107.6 ± 8.7	27.1
2c	22/12/2017	*Velesunio* sp.	MC	45	3.6	17.1 ± 0.5	6.02 ± 0.05	106.4 ± 8.2	27.1
3	29/04/2018	*Velesunio* sp.	GC	75	3.2	12.3 ± 0.6	6.03 ± 0.05	101.9 ± 8.9	26.6
4	15/05/2018	*Velesunio angasi*	NC	100	14.2	16.5 ± 0.8	6.02 ± 0.05	108.9 ± 9.4	26.8
Un-spiked sediment:									
5	6/06/2018	*Velesunio* sp.	MC	95	24.0	14.2 ± 0.4	6.02 ± 0.05	110.5 ± 7.9	27.2
6	12/06/2018	*Velesunio angasi*	SB	100	28.4	15.7 ± 0.4	6.01 ± 0.07	112.4 ± 9.1	27.1
7	4/08/2018	*Velesunio angasi*	SB	100	28.1	15.7 ± 0.6	6.09 ± 0.11	110.6 ± 10.2	27.2
		Mean ± SD				*15.7 ± 1.9*	*6.04 ± 0.3*	*109.7 ± 4.3*	*27.0 ± 0.2*

[a] Values represent measurements of 2 pooled replicate samples at 0, 2, 4, 6, 8, 10, 12, 14 d from all treatments (Test 1: n = 8, Test 2: n = 11, Tests 3 – 7: n = 8) for each chronic test.

[b] Incubator temperature was monitored at 5-min intervals using data loggers (Testo Saveris™). New test waters were acclimated for 2-3 hours prior to test commencement and water changes, and heat pads were used during transfer of juveniles into new water.

7.3.2 Pre-spiked sediment tests - chemistry (Tests 1 – 4)

Pre-spiking sediments with U did little to reduce the loss from the water column. Figures 7.1 and 7.2 represent U concentrations of filtered and total fractions for all tests using pre-spiked sediment (Tests 1, 2a-c, 3, and 4), and Supplementary Data, Table S7.1 provides further detail of the following results.

7.3.2.1 Filtered fraction

When comparing the water chemistry of filtered concentrations, loss of U was considerable despite pre-spiking the sediment, with average concentrations of filtered U at the start of each test reduced from nominal concentrations by 62 to 79%, and by a further 58 to 70% between the start and end of each test. For Test 2c, where no sediment was used, U loss between nominals and starting concentrations in the filtered fractions was minimal (7.6%), and was also very low between the start and end of test (3.5%). Measured concentrations of filtered U at the end of each test (Tests 1 – 4, excluding Test 2c) ranged from 7.9 to 17.4% of total U.

7.3.2.2 Total fraction

Average U concentrations of total fractions were more variable than those of filtered fractions, ranging from a loss of 22% to an increase of 22% from nominal concentrations, and a loss of 14% to an increase of 37% between start and end concentrations. Uranium concentrations varied in the total fractions, depending on the sediment spiking method. For Tests 1 and 4 where sediment was pre-spiked according to the nominal test concentrations and overlying water was removed, a similar overall reduction in total U concentrations occurred (10 and 6% respectively). For Test 2a, where pre-spiked sediment was washed three times, concentrations of total fractions were variable, ranging between a 12% loss in the highest concentration, to a 38% increase in the lowest concentration. For Test 3, where the overlying water was not removed, an increase was seen (up to 57%) in all except one treatment (where there was a loss of 4%). For Test 2b, where sediments were spiked to 1×, 2×, 4×, and 8× nominal U concentrations, U concentrations increased in total fractions in all treatments by an average of 37% (ranging 29 – 53%). For Test 2c, where no sediment was used, U loss between nominals and starting concentrations in the total fractions averaged 6.5%, and was 3.5% between the start and end of test.

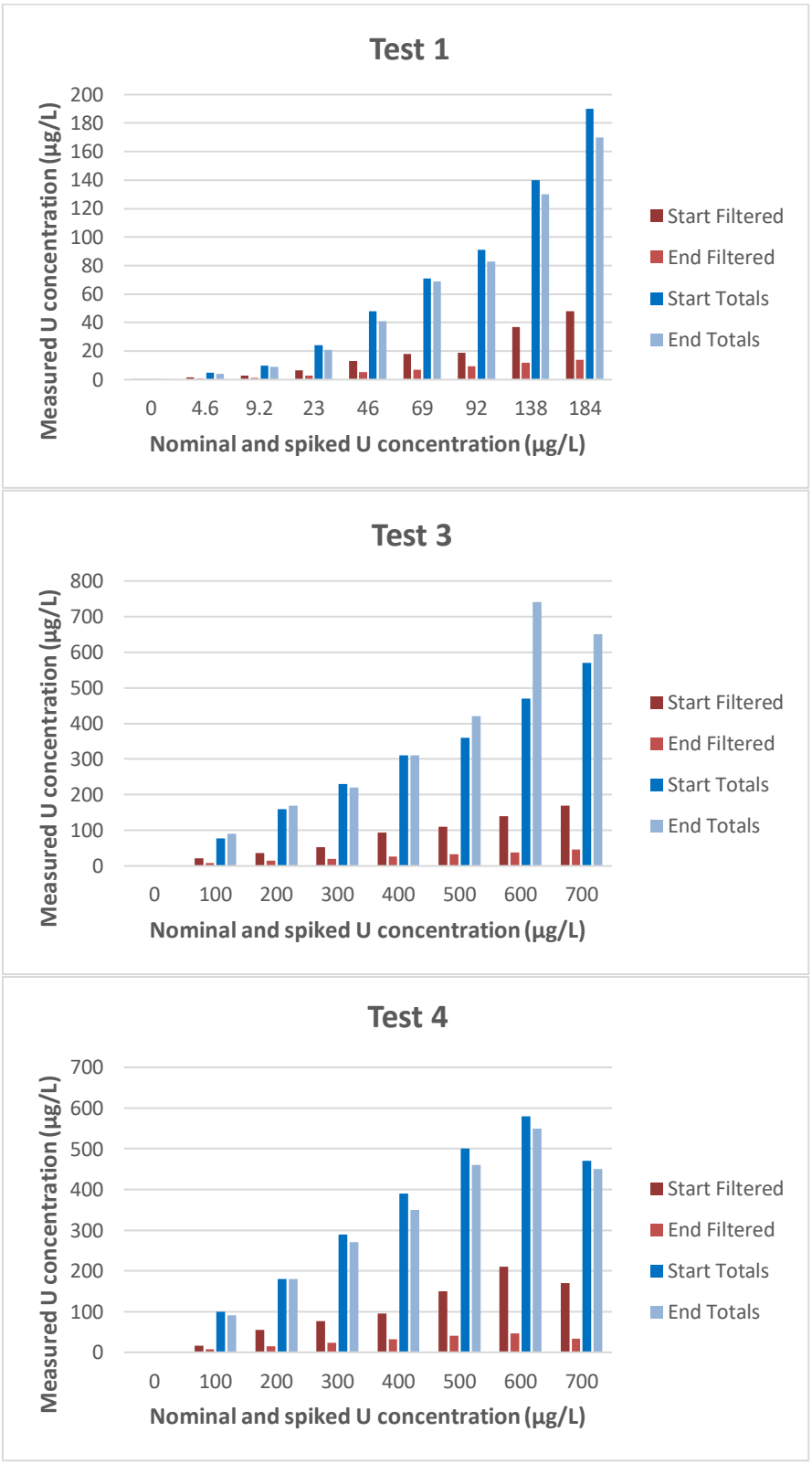

Figure 7.1: Water chemistry results for Tests 1, 3, and 4, where sediment was spiked to nominal concentrations, and overlying water after spiking was (Test 1): Removed and replaced with MilliQ, (Test 3): Not removed, and (Test 4): Removed and replaced with MCW (diluent water).

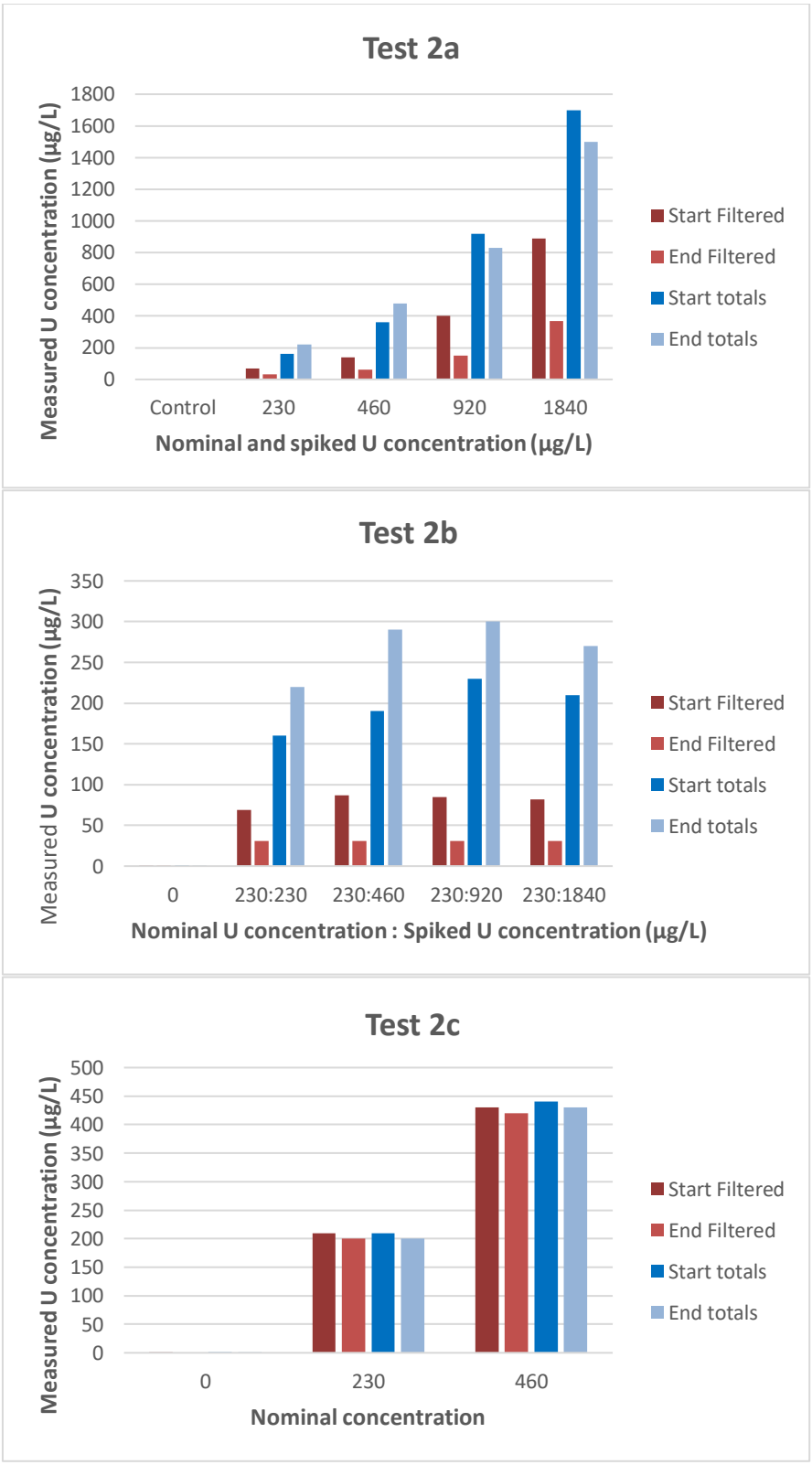

Figure 7.2: Water chemistry results for Tests 2a, 2b, and 2c, where sediment was (Test 2a): Spiked to nominal concentrations and washed 3× in MilliQ, (Test 2b): Spiked to 1×, 2×, 4×, and 8× nominal concentrations and washed 3×, and (Test 2c): Not used.

7.3.3 Un-spiked sediment tests - chemistry (Test 5 – 7)

Using un-spiked sediment in toxicity tests (Tests 5 to 7) did not result in significantly greater loss of U from the water column than tests using pre-spiked sediment, and changes were less variable in both the filtered and total fractions (Figure 7.3, and Supplementary Data, Table S7.1).

7.3.3.1 Filtered fraction

Loss of U in the filtered fraction was similar at the start of Tests 5 to 7 to that of tests 1 to 4 using pre-spiked sediment, with concentrations reduced at the start of each test by 63 – 76% from nominals. Further average loss of the filtered U fraction between the start and end of tests was slightly higher than tests 1 to 4, ranging from 74 – 84%. Measured concentrations of U in the filtered fraction at the end of each test ranged from 5.9 to 9.8% of U concentrations in the total fraction.

7.3.3.2 Total fraction

Concentrations of total fractions in each test experienced an overall average loss from nominal concentrations ranging from 7 to 21%, and a further loss ranging from 1 to 14% between the start and end of tests. This was a less variable change when compared to Tests 1 to 4 using pre-spiked sediment). Generally, there was a greater and more variable loss in the total fraction for Test 7.

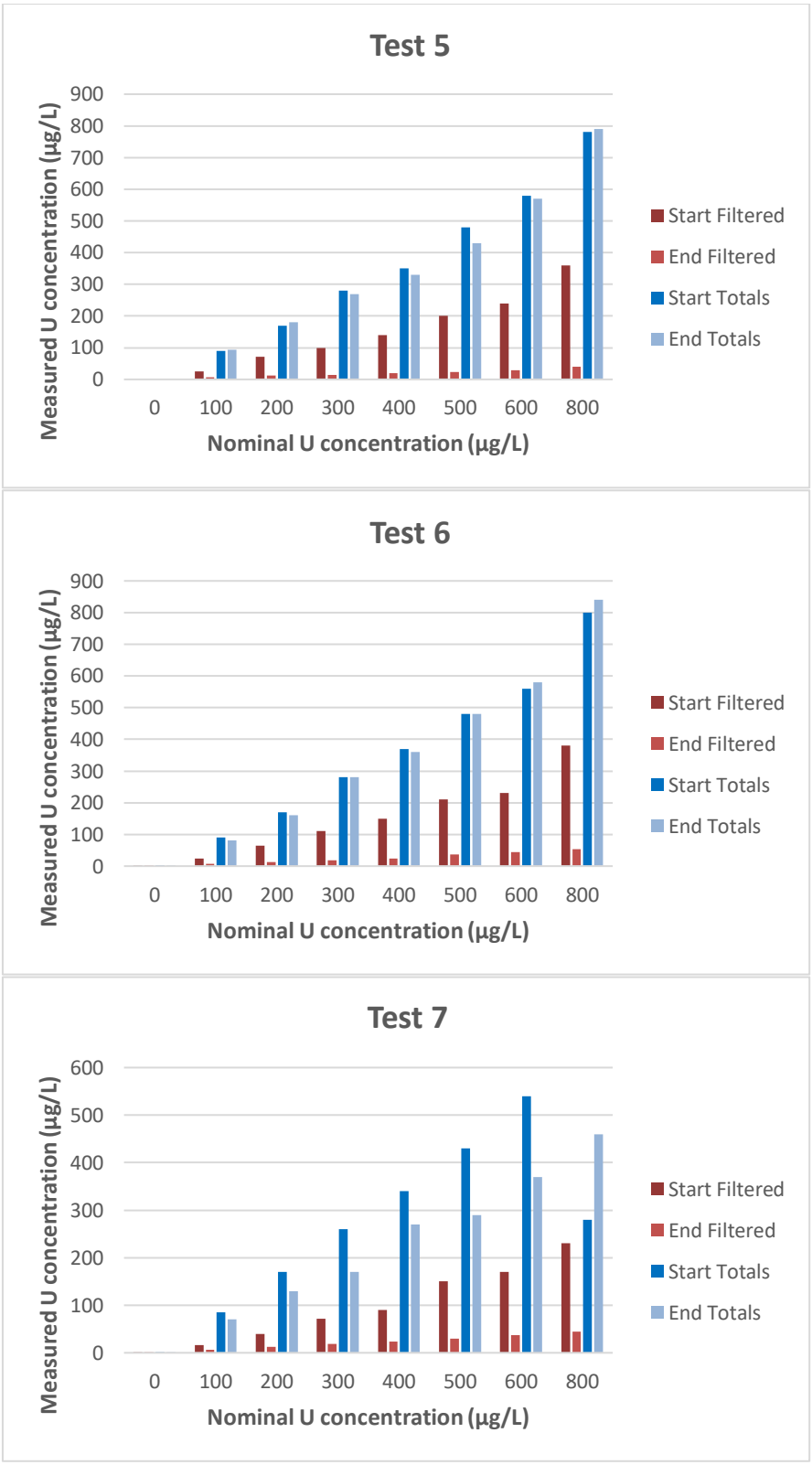

Figure 7.3: Water chemistry for Test 5, Test 6, and Test 7, where sediment was not pre-spiked with U, showing differences in measured filtered and total U concentrations between the start and end of each test.

7.3.4 Juvenile control growth and survival

The different sediment spiking methods resulted in varying control survival and growth. Control survival was acceptable (≥ 80%) in all tests except for Test 2c (without sediment; 45%) and Test 3 (75%). Tests 1 and 4 both achieved similar control growth (14.1 and 14.2 µm/day respectively) which was considered low, but within the acceptable range of 13–32 µm/day (Table 7.5). In Test 2a, where pre-spiked sediment was washed three times, growth rate was greatly reduced and unacceptably low in all treatments, achieving only 6.6 µm/day in the control, and 4.5 and 1.8 µm/day in the two other treatments that survived. In Test 2b, where sediments were spiked to 1×, 2×, 4×, and 8× nominal U concentrations and sediment was washed three times, a similar reduction in growth was achieved (ranging 59 – 69% of control growth) in all treatments regardless of the sediment spike concentration (2×, 4×, or 8×). However, growth rate was greatly reduced and all treatments, achieving only 6.6 µm/day in the control, and between 3.9 to 4.5 µm/day in other treatments. Similarly, in Test 3, where the overlying water was not removed, growth was greatly reduced and unacceptably low in all treatments, achieving only 3.2 µm/day in the control, and between 1.3 to 2.9 µm/day in other treatments. For Test 2c, where no sediment was added, control growth was reduced to 55% of the control treatment containing sediment, and juveniles in the test treatments did not survive beyond eight days. Together with the very low control survival of 45%, this test demonstrated the importance of sediment in the test protocol as an additional nutritional source for optimal growth and survival. Valenti *et al.* (2005) observed similar mortality (>50%) in control treatments without sediment after seven days, despite feeding and water changes. Tests 5 to 7 using un-spiked sediment achieved excellent control survival (95 – 100%) and growth (24.0 to 28.4 µm/day), within acceptable parameters.

7.3.5 Chronic uranium toxicity

The growth data from five of the seven tests produced good concentration-response gradients in both the filtered (<0.45 µm) and total fractions (Figures 7.4 and 7.5). The individual 14-d EC50s based on the filtered fractions (representing water-borne exposure) ranged from 64 to 126 µg/L, a 2-fold variation, and the EC10s ranged from 14 to 50 µg/L, a 3.6-fold variation (Table 7.6). For the total fractions (representing water-borne and dietary exposure), individual 14-d EC50s ranged from 276 to 506 µg/L, a 1.8-fold variation, and were between 4 and 5.7-fold higher than those based on the filtered fractions. The EC10s

ranged from 64 to 319 µg/L, a 5-fold variation, and were between 3.9 and 6.4-fold higher than those based on the filtered fractions (Table 7.6).

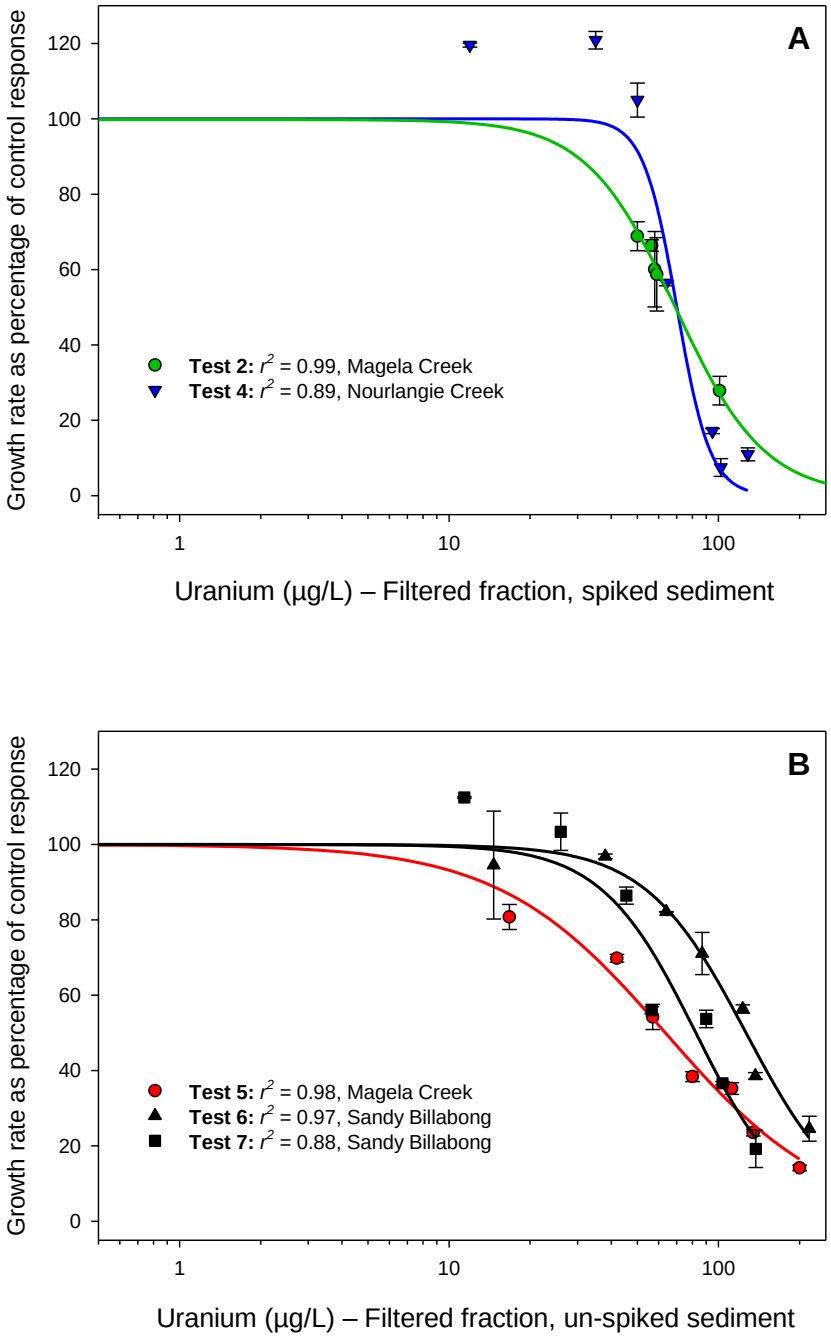

Figure 7.4: Uranium concentration-response relationships for chronic 14-d exposures to *Velesunio* spp. juveniles with (A) pre-spiked sediment, and (B) un-spiked sediment, using the average measured filtered (0.45 µm) U fraction. The 95% confidence intervals have been removed for clarity. Data points represent each of two replicates per treatment. Toxicity estimates were determined using 3-parameter logistic models.

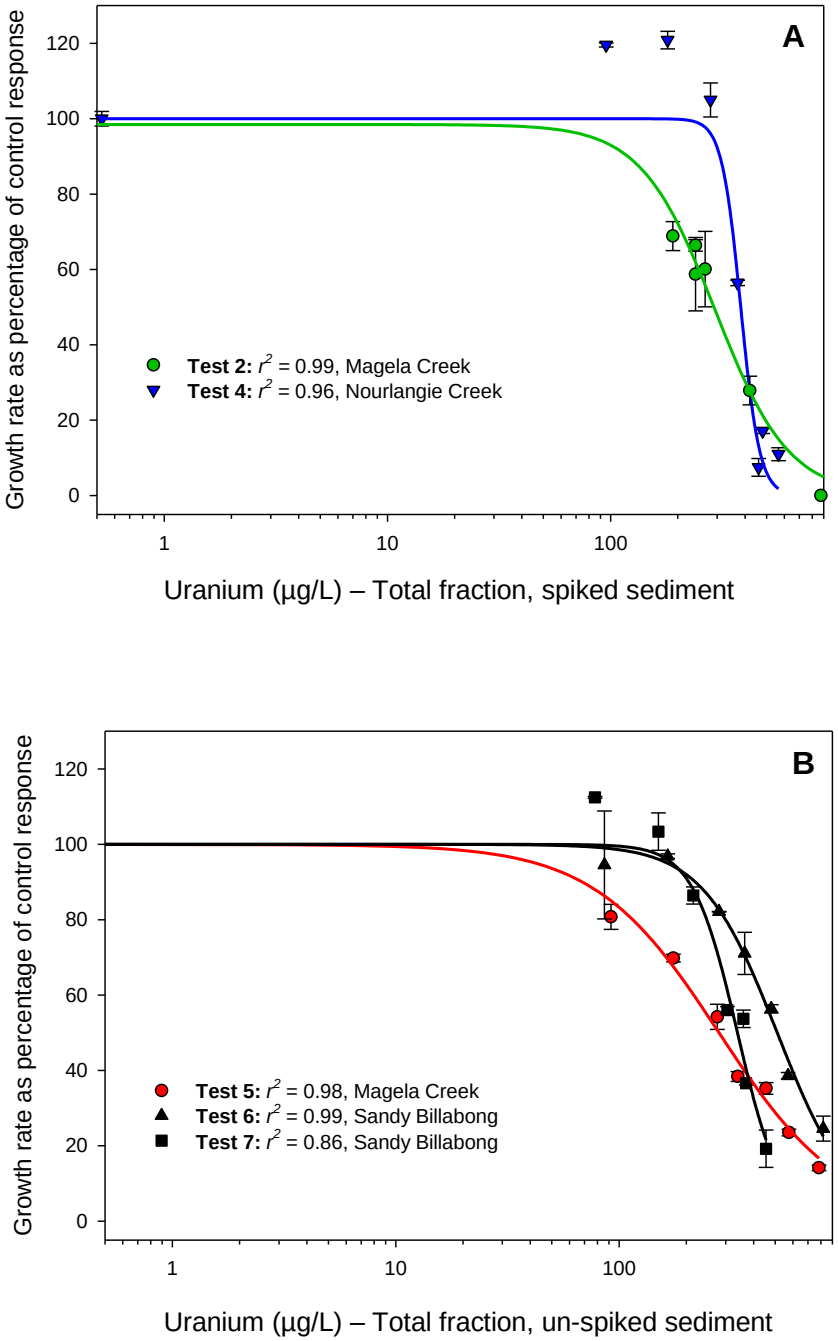

Figure 7.5: Uranium concentration-response relationships for chronic 14-d exposures to *Velesunio* spp. juveniles with (A) pre-spiked sediment, and (B) un-spiked sediment, using the average measured total U fraction. The 95% confidence intervals have been removed for clarity. Data points represent each of two replicates per treatment. Toxicity estimates were determined using 3-parameter logistic models.

Table 7.6: Summary of the chronic 14-d tests with *Velesunio* spp. juveniles showing the U toxicity estimates (95% CI) using measured filtered 0.45 µm fraction concentrations, and measured total fraction concentrations. Tests 1 – 4 included U-spiked sediment, and tests 5 – 7 included un-spiked sediment. SB = Sandy Billabong, MC = Magela Creek, GC = Gulungul Creek, NC = Nourlangie Creek.

			Filtered 0.45 µm fraction		Total fraction	
Test	Species	Site	EC50 (µg/L)	EC10 (µg/L)	EC50 (µg/L)	EC10 (µg/L)
U-spiked sediment:						
1 [a]	*Velesunio angasi*	SB	-	-	-	-
2a+b [b]	*Velesunio* sp.	MC	69 (66 – 73)	30 (23 – 35)	286 (247 – 330)	118 (n/a – 175)
3 [b]	*Velesunio* sp.	GC	-	-	-	-
4	*Velesunio angasi*	NC	69 (51 – 96)	50 (n/a – 64)	391 (361 – 431)	319 (220 – 357)
Un-spiked sediment:						
5	*Velesunio* sp.	MC	64 (54 – 76)	14 (3 – 22)	276 (244 – 312)	64 (26 – 96)
6	*Velesunio angasi*	SB	126 (113 – 140)	48 (29 – 62)	506 (473 – 542)	215 (165 – 254)
7	*Velesunio angasi*	SB	81 (61 – 109)	33 (n/a – 53)	347 (287 – 427)	199 (n/a – 277)

[a] The U concentration range was too low to get a full effect for deriving EC50/EC10s
[b] Control growth did not meet minimum requirements, due to sediment-spiking reducing growth in all treatments.

7.4 Discussion

7.4.1 Water chemistry

Pre-spiking the sediment with U concentrations was not effective in minimising loss of U to the sediment. In addition, pre-spiking produced more variation in U concentrations in the total fractions and an overall reduction in juvenile growth rates and survival, when compared to tests with un-spiked sediment.

Based on the similar results of Tests 1 and 4, small differences in sediment spiking methods such as spiking wet or dry sediment, heating samples after spiking, or replacing overlying water with MCW versus MilliQ, did not markedly influence the water chemistry, survival, and growth. Removal of the overlying water after sediment spiking may have removed DOC from the sediment, making it unavailable as a food source, thus resulting in reduced growth. A reduction in growth was even more evident in Tests 2a and 2b, when the sediment was washed three times. Washing the sediment may have removed a greater amount of DOC, reducing growth in both the controls and test treatments. Spiking the sediments only to the desired nominal U concentrations may have resulted in different proportions of binding sites being occupied, unless even the lowest concentration was sufficient to saturate all the U binding sites. Specific spiking optimization studies where the sediment is spiked at different U concentrations and the concentration of U adsorbed to the sediment is measured could show if an asymptote is observed.

Spiking the sediment to 2×, 4×, and 8× the test water concentration (Test 2b) did not affect the loss in the filtered U concentrations, which achieved similar U loss to test 2a. However, total U concentrations increased by an average of 37% between the start and the end of the test as a result of the sediment spiking. The over-spiking did not result in higher than nominal Total U concentrations, possibly because U binding sites in sediment particles had reached saturation, and triple-washing may have removed excess U that did not bind to the sediment or form U complexes in the water. Test 3 achieved the lowest control survival (75%) and growth (3.2 μm/day) of all tests with pre-spiked sediment, and was the only test in which the overlying water of the spiked sediment sample was not removed. For this test, concentrations of U in the total fraction increased by an average of 22% between the start and the end of the test, in contrast to Tests 1 and 4 where U concentrations decreased by 10% and 7% respectively. Mobilised U retained in the overlying water of the spiked sediment sample may have formed complexes with the DOC contained in the test diluent (MCW), reducing the availability of DOC to the mussels as a food source. The effect of U complexation with DOC

in reducing U bioavailability and toxicity to other tropical freshwater organisms has been demonstrated in other studies (Hogan *et al*. 2005; Trenfield *et al*. 2011b), but is unknown for freshwater mussel early life stages. Measurement of the DOC throughout the experiments was not carried out, but may be important to characterize due to its importance for U bioavailability and mussel nutrition.

When no sediment was used in the test system (Test 2c), juvenile mussel growth and survival were reduced to unacceptable levels, demonstrating the need for sediment as an additional food source. In this test, U loss between nominal and starting concentrations in the filtered and total fractions was minimal (7.6 and 6.5% respectively), and reflects the extent of loss to the test apparatus. Loss was also minimal (3.5%) between the start and end of the test in both the filtered and total fractions, demonstrating that the majority of U loss in the tests using added sediment can be attributed to the sediment, rather than to the test apparatus (Figure 7.2).

7.4.2 Chronic uranium toxicity

The estimated EC50s and EC10s varied depending on whether U concentrations in the filtered or total U concentrations were used for the analysis. In Australia and New Zealand, total metal concentrations can be used to evaluate toxicity data, but generally the filtered (0.45 µm) metal fraction is used for GV derivation and comparison of data, as it is more representative of metal bioavailability to organisms (ANZG 2018). However, the water chemistry results of this study, and our knowledge of the organism's physiology (the extent to which it ingests, and would have contact with U-contaminated sediment), suggest that a significant part of the toxic response was through dietary U exposure, and that total U concentrations may be more appropriate to use for comparison in this case.

A significant amount of metal exposure may occur through the mussel diet (Cope *et al*. 2008), which in this study included the added sediment and algae. Besides the added sediment, it is possible that juveniles were exposed to toxic effects through U binding to their live algal diet. However, the results of Test 2c (without sediment) indicate that the amount of U binding to the algae was minimal in comparison to the sediment, with the total loss between nominal and end filtered concentrations measuring ~10%, which included loss to the test apparatus, algae and mussels. In contrast, the loss between nominal and end filtered concentrations in pre-spiked tests 1, 2a, 2b, 3, and 4 ranged between 84 to 93%, and in un-spiked tests 5-7 ranged between 94 to 95%, representing the amount of U loss to the test

apparatus, algae, mussels and sediment, suggesting that between 74 to 85% of U was rapidly bound to the sediment.

The low pH, low hardness, and low DOC of the test waters is likely to have increased the bioavailability and therefore toxicity of dissolved U to the mussels. This has been demonstrated in a study by Croteau *et al*. (2016), who characterised the bioavailability of dissolved U over ranges of hardness and pH, and in the presence of DOC, to the freshwater snail, *Lymnaea stagnalis*. They found that dissolved U was bioavailable under all conditions tested, and bioavailability decreased with increasing pH, Ca and Mg concentrations in the presence of DOC. A study by Roditi *et al*. (2000) found that the absorption of DOC in natural waters by zebra mussels (*Dreissena polymorpha*) could contribute up to 50% of their carbon requirements, and that absorption of some dissolved metals that had been complexed with DOC increased in varying amounts when DOC was present.

A large amount of research has been undertaken for the development and revision of site-specific GVs for key contaminants from the Ranger mine, utilising chronic data that has been generated for local freshwater species (van Dam *et al*. 2017). In comparison with chronic data (EC50s ranging between 114 – 1435 µg/L U) for seven other freshwater taxa tested in soft waters of similar physico-chemical composition, the EC50s for *Velesunio* sp. and *V. angasi* were lower (64 and 101 µg/L U) when the filtered U fraction was used, and within the range (276 and 419 µg/L U) of EC50s for other species when the total U fraction was used, suggesting a moderate sensitivity to uranium (Table 7.7). Based on the results, the current site-specific GV for U (2.8 µg/L) would be protective for *Velesunio* spp. surrounding the mine.

Table 7.7: Data comparisons for U toxicity derived in soft waters of similar physico-chemistry.

Species	**Test type**	**EC50 (ug/L)**	**EC10 / NOECs (ug/L)**	**Reference**	**Value used for revised site-specific limit** [a] (van Dam *et al*. 2017)
Moinodaphnia macleayi Cladoceran	3 brood reproduction	160 - 390	8 – 31 (NOECs)	Semaan *et al*. (2001)	16
Amerianna cumingi	96-h embryo production	278	15	Hogan *et al*. (2010);	17
Snail	14-d embryo production	153	46	Mooney *et al*. (2016)	
Ceratophyllum demersum	168-h growth inhibition	134 – 547 [b]	29 – 121 [b] (NOECs)	Markich (2013)	34
Hydra viridissima Green hydra	96-h population growth	114 – 219 [c]	14 – 81 [c]	Riethmuller *et al*. (2000)	47
Chlorella sp. Green alga	72-h cell division rate	137–238	72-157 (NOECs)	Hogan *et al*. (2005)	49
Lemna aequinoctialis Duckweed	96-h plant growth rate	1435	207	Hogan *et al*. (2010)	200
Mogurnda mogurnda	28-d growth (dry weight)	>1400 / 1130	860 / 660	Cheng *et al*. (2010)	573
Fish	28-d growth (length)	>1400 / >1200	1160 / 850		
Velesunio sp. Mussel	14-d growth rate	64 / 276 [d]	14 / 64 [d]	This study	-
Velesunio angasi Mussel	14-d growth rate	101 / 419 [d]	40 / 207 [d]	This study	-

[a] Revised limit 2.8 µg/L at 2 mg/L DOC

[b] Hardness ranging from 20 – 400 mg/L $CaCO_3$

[c] Hardness ranging from 6.6 – 330 mg/L $CaCO_3$

[d] Filtered (0.45 µm) U concentration / Total U concentration.

7.4.3 Limitations

From these results, several limitations of the test design are evident, and sediment spiking methods would need to be further investigated and refined if they are to be used in the toxicity test experimental design. Whilst the water chemistry results provided valuable information on the behaviour of U in the test system, a more systematic approach is needed to optimize the sediment spiking procedure. Further studies would more clearly determine the fate of U, and may involve measuring the temporal dynamics of U loss to show when most loss occurred. Taking U measurements at the start and the end of each test effectively reflects the loss that occurred (i) in the storage bottle over 14-d (if at all measurable), and (ii) over the previous 48-h in the test containers, because water changes were performed every 48-h using test water prepared in bulk at the start of the test. Previous research with the freshwater snail, *Amerianna cumingi,* involved extensive characterisation of the fate of U, revealing that the majority of U loss occurred through binding to the test apparatus during the first 4 days of the 14-d test due to initial binding (priming) of U to the test apparatus surfaces (Hogan *et al*. 2010; Mooney *et al*. 2016). However, the presence of sediment in the present study warrants further investigation into the fate of U.

Spiked and un-spiked sediment controls could quantify effects on the mussels due to U bound to the sediment, and a spiked sediment only test with no U added to the test diluent could determine the extent of dietary exposure. Further work is needed to better characterise U speciation and its effects on the mussels before the toxicity test method can be used for assessing the chronic toxicity of *Velesunio* spp. with U and other metals.

7.5 Conclusion

The results of the present study demonstrate the importance of evaluating the effects of water chemistry when using sediments in U toxicity tests with juvenile freshwater mussels. The incorporation of the sediment pre-spiking step in the method resulted in reduced control growth, and in some cases, reduced survival. In contrast, toxicity tests without the sediment-spiking step achieved excellent control growth and survival, and the data produced high-quality chronic toxicity estimates. The water chemistry results revealed that a large amount of U was lost to the sediment, and that most of the U toxicity was via the dietary route. For this reason, toxicity estimates using the total measured U fraction may be a better indicator of

effects when using the data for deriving or comparing to GVs for uranium. Limitations in the experimental design suggest a need for further work in characterising U speciation in the test system, to better determine the effects of U on the mussels. The chronic U data presented in this study represents the first for juveniles of two tropical freshwater mussel species; the lentic *Velesunio angasi* and the lotic *Velesunio* sp. Chronic toxicity estimates indicated that both species were moderately sensitive to uranium in comparison with other tropical freshwater taxa exposed to U in soft water of similar composition, although additional data is required to test this hypothesis.

Supplementary Data

Table S7.1: Results of measured 0.45 µm filtered fractions and total U fractions in each test concentration for each 14-d chronic U test. All dissolved samples were filtered to 0.45 µm. SB = Sandy Billabong, MC = Magela Creek, GC = Gulungul Creek, NC - Nourlangie Creek.

				0.45 µm filtered U concentrations (µg/L)				Total U concentrations (µg/L)				Growth		
Test, site	Treatment	Nominal Conc.	Sediment Spike Conc.	Start	End	% change start/nom	% change start/end	Start	End	% change start/nom	% change start/end	Survival (%)	(av. µm/day)	(% of control)
1.	A - Control	0	0	0.023	0.04			0.24	0.29			95	14.1	100.0
SB	B	4.6	4.6	1.7	0.65	-63.0	-61.8	4.8	4	4.3	-16.7	100	15.5	109.9
	C	9.2	9.2	3	1.3	-67.4	-56.7	9.6	8.9	4.3	-7.3	100	16.4	116.1
	D	23	23	6.6	3	-71.3	-54.5	24	21	4.3	-12.5	85	17.8	126.3
	E	46	46	13	5.1	-71.7	-60.8	48	41	4.3	-14.6	95	18.3	129.5
	F	69	69	18	6.8	-73.9	-62.2	71	69	2.9	-2.8	100	17.6	124.9
	G	92	92	19	9.3	-79.3	-51.1	91	83	-1.1	-8.8	100	15.7	111.3
	H	138	138	37	12	-73.2	-67.6	140	130	1.4	-7.1	90	12.8	90.9
	I	184	184	48	14	-73.9	-70.8	190	170	3.3	-10.5	100	9.4	66.3
			Mean			*-71.7*	*-60.7*			*3.0*	*-10.0*			
2a.	A - Control	0	0	0.017	0.41			0.19	0.74			90	6.6	100.0
MC	B	230	230	69	31	-70.0	-55.1	160	220	-30.4	37.5	90	4.5	68.9
	C	460	460	140	62	-69.6	-55.7	360	480	-21.7	33.3	85	1.8	27.9
	D	920	920	400	150	-56.5	-62.5	920	830	0.0	-9.8	0	0.0	0.0
	E	1840	1840	890	370	-51.6	-58.4	1700	1500	-7.6	-11.8	0	0.0	0.0
			Mean			*-61.9*	*-57.9*			*-14.9*	*12.3*			
2b.	Control	0	0	0.017	0.41			0.19	0.74			90	6.6	100.0
	B	230	230	69	31	-70.0	-55.1	160	220	-30.4	37.5	90	4.5	68.9
	F	230	460	87	31	-62.2	-64.4	190	290	-17.4	52.6	85	3.9	58.8
	G	230	920	85	31	-63.0	-63.5	230	300	0.0	30.4	85	4.0	60.1
	H	230	1840	82	31	-64.3	-62.2	210	270	-8.7	28.6	90	4.4	66.4
			Mean			*-64.9*	*-61.3*			*-14.1*	*37.3*			
2c.	Control	0	-	0.23	0.001			0.23	0.4			45	3.6	55.0
	I	230	-	210	200	-8.7	-4.8	210	200	-8.7	-4.8	0	0.0	0.0
	J	460	-	430	420	-6.5	-2.3	440	430	-4.3	-2.3	0	0.0	0.0
			Mean			*-7.6*	*-3.5*			*-6.5*	*-3.5*			

Table S7.1 continued…

Test, site	Treatment	Nominal Conc.	Sediment Spike Conc.	0.45 µm filtered U concentrations (µg/L)				Total U concentrations (µg/L)				Growth		
				Start	End	% change start/nom	% change start/end	Start	End	% change start/nom	% change start/end	Survival (%)	(av. µm/day)	(% of control)
3.	A - Control	0	0	0.02	0.11			0.22	0.49			75	3.2	100.0
GC	B	100	100	22	8.4	-78.0	-61.8	77	91	-23.0	18.2	75	2.9	91.6
	C	200	200	36	15	-82.0	-58.3	160	170	-20.0	6.3	80	2.7	83.4
	D	300	300	53	19	-82.3	-64.2	230	220	-23.3	-4.3	85	1.6	50.4
	E	400	400	93	26	-76.8	-72.0	310	310	-22.5	0.0	75	1.3	39.3
	F	500	500	110	33	-78.0	-70.0	360	420	-28.0	16.7	60	1.8	55.8
	G	600	600	140	38	-76.7	-72.9	470	740	-21.7	57.4	65	1.6	51.2
	H	700	700	170	46	-75.7	-72.9	570	650	-18.6	14.0	60	2.1	65.7
			Mean			*-78.5*	*-67.4*			*-22.4*	*15.5*			
4.	A - Control	0	0	0.006	0.17			0.46	0.59			100	14.2	100.0
NC	B	100	100	16	7.9	-84.0	-50.6	100	91	0.0	-9.0	95	17.0	119.6
	C	200	200	55	15	-72.5	-72.7	180	180	-10.0	0.0	100	17.2	120.9
	D	300	300	77	23	-74.3	-70.1	290	270	-3.3	-6.9	100	15.0	105.0
	E	400	400	96	32	-76.0	-66.7	390	350	-2.5	-10.3	95	8.0	56.5
	F	500	500	150	40	-70.0	-73.3	500	460	0.0	-8.0	85	2.4	17.1
	G	600	600	210	47	-65.0	-77.6	580	550	-3.3	-5.2	70	1.6	11.0
	H	700	700	170	34	-75.7	-80.0	470	450	-32.9	-4.3	90	1.1	7.5
			Mean			*-73.9*	*-70.2*			*-7.4*	*6.2*			

Table S7.1 continued…

Test, site	Treatment	Nominal Conc.	Sediment Spike Conc.	0.45 µm filtered U concentrations (µg/L)				Total U concentrations (µg/L)				Growth		
				Start	End	% change start/nom	% change Start/end	Start	End	% change start/nom	% change Start/end	Survival (%)	(av. µm/day)	(% of control)
5. MC	A - Control	0	-	0.023	0.15			0.27	0.48			100	23.9	100.0
	B	100	-	26	7.5	-74.0	-71.2	90	94	-10.0	4.4	85	19.3	80.8
	C	200	-	72	12	-64.0	-83.3	170	180	-15.0	5.9	90	16.7	69.8
	D	300	-	100	14	-66.7	-86.0	280	270	-6.7	-3.6	100	13.0	54.2
	E	400	-	140	20	-65.0	-85.7	350	330	-12.5	-5.7	100	9.2	38.4
	F	500	-	200	24	-60.0	-88.0	480	430	-4.0	-10.4	95	8.4	35.2
	G	600	-	240	29	-60.0	-87.9	580	570	-3.3	-1.7	95	5.6	23.5
	H	800	-	360	40	-55.0	-88.9	780	790	-2.5	1.3	95	3.4	14.1
			Mean			*-63.5*	*-84.4*			*-7.7*	*-1.4*			
6. SB	A - Control	0	-	0.019	0.069			0.2	0.35			100	28.4	100.0
	B	100	-	23	6.3	-77.0	-72.6	90	82	-10.0	-8.9	85	26.9	94.5
	C	200	-	64	12	-68.0	-81.3	170	160	-15.0	-5.9	100	27.5	96.9
	D	300	-	110	18	-63.3	-83.6	280	280	-6.7	0.0	100	23.3	82.1
	E	400	-	150	24	-62.5	-84.0	370	360	-7.5	-2.7	100	20.2	71.1
	F	500	-	210	36	-58.0	-82.9	480	480	-4.0	0.0	95	16.0	56.4
	G	600	-	230	44	-61.7	-80.9	560	580	-6.7	3.6	100	11.0	38.6
	H	800	-	380	54	-52.5	-85.8	800	840	0.0	5.0	85	7.0	24.6
			Mean			*-63.3*	*-81.6*			*-7.1*	*-1.3*			
7. SB	A - Control	0	-	0.027	0.16			0.22	0.39			100	28.1	100.0
	B	100	-	16	6.8	-84.0	-57.5	85	71	-15.0	-16.5	100	31.6	112.5
	C	200	-	40	12	-80.0	-70.0	170	130	-15.0	-23.5	100	29.1	103.4
	D	300	-	72	19	-76.0	-73.6	260	170	-13.3	-34.6	100	24.3	86.4
	E	400	-	90	23	-77.5	-74.4	340	270	-15.0	-20.6	100	15.8	56.0
	F	500	-	150	30	-70.0	-80.0	430	290	-14.0	-32.6	100	15.1	53.7
	G	600	-	170	37	-71.7	-78.2	540	370	-10.0	-31.5	100	10.3	36.6
	H	800	-	230	45	-71.3	-80.4	280	460	-65.0	64.3	85	5.4	19.2
			Mean			*-75.8*	*-73.5*			*-21.0*	*-13.6*			

CHAPTER 8: General discussion and conclusions

8.1 Achievement and impact

The research described in this thesis aimed to address the knowledge gaps regarding species identification, toxicity testing methods, and sensitivity to contaminants of tropical freshwater mussels from Northern Australia. The secondary objective of investigating the genetic diversity of freshwater mussel species found throughout tropical NT was achieved using mitochondrial DNA analysis, and revealed genetic differences amongst the sampled sites. The genetic results informed the selection of mussel species used for the primary objectives of this research; to optimise acute and chronic toxicity test methods for tropical freshwater mussels, and to apply the optimised methods in the assessment of key mining contaminants from the Ranger uranium mine.

The genetics study (Chapter 2) was effective in demonstrating the existence of genetic variation among mussels from 13 sampled sites throughout the northern part of the Northern Territory, including at least one undescribed species. Accurate characterisation of the test species was a necessary step for subsequent toxicity test optimisation and toxicity testing, due to the influence of genetic differences on species' tolerance to environmental contaminants. Taxonomic uncertainties may lead to inaccurate management decisions where toxicity data are used for monitoring contaminants or deriving GVs. In particular, this investigation was important in determining if different mussel species existed within the Alligator Rivers Region, an area of high conservation value, which is adjacent to uranium mining activities.

Freshwater mussels are known to exhibit high phenotypic plasticity (Zieritz *et al.* 2018), and molecular techniques such as mitochondrial DNA analysis have proven to be effective in revealing phylogenetic relationships (Wu *et al.* 2019). Previous research on the biology and ecology of the freshwater mussels in the Magela Creek system focused on a described species, *Velesunio angasi*, and several phenotypic variances were observed in the different lotic and lentic waterbodies (Humphrey and Simpson 1985).

In the present study, mussels collected from 13 different sites (creeks, billabongs, and lakes) were assessed genetically using mtDNA analysis. Phylogenetic analysis of 194 generated sequences produced evidence of three distinct clades (A, B, and C) with divergence between clades varying significantly between 10.1 to 11.1%. The results confirmed the presence of *V. angasi* in several locations within and outside of the ARR, and the presence of a new undescribed closely related species found in lotic environments was also revealed. The

latter has been tentatively labelled *Velesunio* sp. throughout this thesis because a detailed taxonomic description of the species was beyond the scope of this thesis. Other known species were also identified from collection sites outside of the ARR, and two further species hitherto undescribed were found. Mussels that were determined suitable for use in toxicity testing were limited to the known species, *V. angasi*, which was found in several accessible sites within the ARR, and was also the only species found in two lentic sites closer to Darwin. The undescribed species, *Velesunio* sp., was also considered suitable for investigation, due to its presence in several lotic (creek) habitats in the vicinity of the Ranger mine lease. Mussels from other sites that were not chosen for this study include those from sites where more than one species was present, or those that were not practical to use due to the location and accessibility of the collection sites, and possible workplace health and safety issues related to crocodile activity.

The optimisation of acute and chronic test protocols described in Chapters 3 and 4 was undertaken using two of the species confirmed through genetic analysis, i.e. the known species *Velesunio angasi,* and the undescribed *Velesunio* sp. The value of freshwater mussels as an ecotoxicological test species has been recognised relatively recently in comparison with that of other test species such as fish (Augspurger *et al.* 2007). While standardised toxicity testing methods existed for the early life stages of freshwater mussels (ASTM 2006), these had been developed using predominantly North American and European temperate mussel species; in response to evidence of their sensitivity to some chemical stressors such as ammonia and copper, and their increasing imperilled conservation status (Augspurger *et al.* 2007). Acute toxicity studies indicated that temperate freshwater mussel glochidia and juveniles were among the most sensitive aquatic species to ammonia (Wang *et al*. 2007a; Clearwater *et al*. 2014) and copper (Jacobson *et al*. 1997; Gillis *et al*. 2008). The use of the standardised toxicity testing protocols has increased the amount of quality toxicity data available for developing water quality criteria in many jurisdictions, particularly in the USA, the world's largest hotspot for freshwater mussel diversity and conservation efforts for endangered species. However, relatively few toxicity studies have been undertaken for freshwater mussel species from the Southern Hemisphere, and there is a distinct lack of data for tropical freshwater mussel species.

Uncertainties about the suitability of several aspects of the standardised methods for tropical mussel species led to the test optimisation experiments described in Chapters 3 to 4 and Appendices B and C. Refinements to the acute toxicity test method were necessary due to several factors including site-specific water characteristics, mussel biology in the tropical climate, and difficulty of mussel collection. For example, a need for pH control was identified, particularly for ammonia tests where toxicity is known to increase with increasing pH. The limited availability of glochidia and the short life span of glochidia in tropical waters, were the other main factors that required addressing in the acute test protocol (Chapter 3). The juvenile mussels used for chronic toxicity testing were acquired in the laboratory by exposing glochidia to host fish, which required an investigation of a suitable host fish, and optimisation of a host-fish exposure method (Chapter 4). Several host fish candidates were selected, based on previous research (Humphrey and Simpson 1985), and availability. The host fish exposure method was refined by adjusting the variables of exposure (amount of glochidia used, number of fish, time of exposure), and conditions of holding fish during the ~10-d mussel encystment period. The obligate parasitic stage is crucial for transformation of glochidia into juvenile mussels for most freshwater mussel species (Kat 1984). While some mussel species have been shown to have varying levels of host specificity (Douda 2015), *Velesunio angasi* appear to be non-species specific (Humphrey and Simpson 1985), although the host fish experiments in this study indicated that the inactive benthic-dwelling gudgeon, *Mogurnda mogurnda*, were more suitable as host fish, as they produced larger and healthier numbers of juveniles than the highly active rainbowfish, *Melanotaenia splendida inornata.*

The standardised chronic test method described in the ASTM (2006) guide was less defined than the acute method, and the need for refinement has been recognised by other researchers (Wang *et al*. 2011). Based on Australian and New Zealand guidance (Warne *et al*. 2018), a shorter test duration of 14 days was selected for the chronic tests in this study using tropical mussel species, rather than the 28-day test duration most commonly used for temperate mussel species (Wang *et al*. 2007c). As shown in previous research (Hudson and Isom 1984), the use of fine sediment as an additional food source proved necessary for optimal growth and survival of juvenile mussels during chronic toxicity tests. Optimisation tests determined the optimal amount of sediment and algae required in the test system, water change frequency and test volume.

The optimised acute and chronic toxicity test methods were successfully applied to *Velesunio* spp., providing valuable and robust toxicity data for the contaminants of interest in waters of low ionic strength. The contaminants investigated are associated with mining practices both nationally and internationally, and had not previously been assessed for the sensitive early life stages (glochidia and juveniles) of tropical freshwater mussels. Therefore, the data generated from this study provides valuable new ammonia, magnesium, copper and uranium toxicity data for tropical mussel species, which may be used for sensitivity comparisons and environmental management purposes.

The effects of contaminants to each life stage appear to be specific to the contaminant tested. For ammonia toxicity assessments (Chapters 3 and 4), both species, *Velesunio angasi* and *Velesunio* sp., were found to be highly sensitive to ammonia in comparison with temperate mussel species and other tropical taxa. Magnesium toxicity assessments (Chapter 5) indicated that both species were moderately sensitive to magnesium when compared to other tropical taxa tested in waters of low ionic strength. Mean LC50s for glochidia and mean EC50s for juveniles were similar for both ammonia and magnesium. Acute copper assessments (Chapter 6) revealed that both species were highly sensitive to Cu, and similarly sensitive in comparison with temperate mussel species. Acute and chronic U assessments (Chapters 6 and 7) showed that *Velesunio* spp. were moderately sensitive when compared with other tropical taxa tested in waters of low ionic strength. Technical challenges were found when the chronic toxicity test was used to assess the toxicity of a metal (i.e. U). Metals generally absorb to the surface of sediment particles, which results in a reduction of dissolved and bioavailable metal concentrations in the water column. The use of pre-spiked sediment in chronic U assessments (Chapter 7) did not significantly reduce the loss of U from the water column, and water chemistry measurements indicated that a large amount of U was sorbed to the silt/sediment. In addition, silt was required by the juvenile mussels for optimal health and survival during the tests, therefore it is likely that significant U exposure occurred via the dietary route. The results provided insight and opportunities for further investigation into dietary routes of exposure for juvenile mussels, which may be more realistic in relation when assessing metal exposure. The analysis of silt/sediment composition could also be included in the standard test method, to provide further information on routes of exposure.

In all toxicity assessments, intra- and interspecies variability in sensitivity for both glochidia and juveniles was low (≤2-fold), and comparable to the variability in other studies assessing metals and salts (Raimondo *et al*. 2016). This indicated that the sensitivity of *Velesunio angasi* and *Velesunio* sp. to the tested contaminants was not significantly different,

and that future toxicity assessments using either species may be undertaken with confidence in similar protection outcomes. The current site-specific water quality GVs for ammonia, magnesium, and uranium in Magela and Gulungul Creeks would be protective of *Velesunio* spp. The standardised toxicity tests may also be used to derive toxicity estimates for other contaminants of potential concern from the Ranger mine, which have been assessed as lower risk to the environment, e.g. manganese, aluminium, cadmium, chromium, and sulfate.

8.2 Limitations and future work

This research has demonstrated that tropical freshwater mussels are a relevant and suitable toxicity test species, and that high-quality toxicity data can be produced using the optimised acute and chronic toxicity test methods. While the research aims have been achieved, limitations have also been identified in using *Velesunio* spp. as a toxicity test organism.

The results of the preliminary genetics study highlighted that genetic differences between mussels in tropical Northern Australia may be more prevalent than previously thought. Mitochondrial DNA is a widely used genetic marker for phylogenetic analysis, since it is relatively easy to amplify (Fernandez-Perez *et al.* 2018), represents maternal inheritance and does not undergo recombination (Zhang and Hewitt 2003). However, limitations exist when using only mtDNA for phylogenetic analysis of mussels. Firstly, the validity of using mtDNA has been questioned due to its use of a single locus (Inoue *et al*. 2018). Secondly, the phenomenon of doubly uniparental inheritance (DUI) of mtDNA in some Unionidae species may confound results (Breton *et al*. 2007), although this has not been reported for the Hyriidae. It is well recognised that a more holistic approach incorporating a combination of markers across the genome (e.g. mtDNA, nuclear DNA, microsatellites, single nucleotide polymorphisms) with morphometric methods can provide a more robust estimation of genetic variation within and between mussel populations (Baker *et al*. 2004; Whelan *et al*. 2011; Inoue *et al*. 2018). Recent technological advances point towards nuclear DNA analyses becoming more routine practice for population-genetic studies (Zhang and Hewitt 2003).

Further research is needed to comprehensively evaluate the population structure and diversity of the freshwater mussels of tropical Northern Australia (Walker *et al.* 2014), particularly mussels in areas of high conservation value such as the ARR. Whilst the phenotypic plasticity in shell morphology of populations in this region has been long recognised, the mtDNA analyses described in this study is the first molecular approach

undertaken for mussels in the ARR, and provides an informative first assessment of mussel species relationships. Further in-depth study incorporating morphological and molecular techniques will provide information on the taxonomic status of mussels throughout the region, which will aid in effective future conservation strategies. In addition, the unknown species identified during this study are strong candidates for formal species description using an integrative morphological and molecular approach.

While culturing methods for freshwater mussels have been successfully developed internationally (ASTM 2006; Barnhart 2006), attempts at culturing adult mussels in the laboratory during this study proved to be impractical and resource intensive. Although adult mussels were housed successfully in the laboratory for up to nine months with little mortality during this study, a lack of glochidia production beyond that of initial post-collection indicated that developing culturing methods for *Velesunio* spp. required further research beyond the scope of this study. Culturing juvenile mussels in the laboratory was a low priority when reliable and accessible mussel collection sites were available for sourcing an initial supply of glochidia from the required mussel species. While *Velesunio angasi* may be collected year-round from several billabong sites, reproduction tends to occur more reliably during the wet season, and collection is hampered by access and safety issues. *Velesunio* sp. are only available during the wet season when creeks are flowing, and the windows of opportunity for collection are very narrow, usually limited to the very start or at the end of the wet season when creek levels are <1.5m, and of low flow. These conditions are necessary for safe access to mussels, and for minimising crocodile risk. Further research is needed to identify reliable, safe, and accessible collection sites for *Velesunio* sp. if they are to be used as a toxicity test species.

The acute toxicity test method used in this study proved to be efficient and reliable in providing toxicity estimates for glochidia. Because fewer glochidia were used than in the standardised ASTM (2006) method, this method could be valuable in toxicity assessments where adult mussels are in short supply or endangered. Minor changes to the method may be required when using temperate species, such as increasing the test duration to 48-h, and decreasing the test temperature. The main limitations of the chronic toxicity test method involved the addition of fine sediment in the test system as an extra nutritional source. Sediment was found to be a necessary test component for optimal juvenile growth and survival, and this was supported by other researchers who found that water-only tests with juveniles produced inconsistent growth and survival (Bringolf *et al*. 2007; Wang *et al*. 2007c, 2010, 2011). However, metal adsorbing to the sediment in the chronic U tests, and markedly

reducing the water-dissolved and bioavailable metal fraction (Chapter 7), is likely to occur in chronic tests with other metals. Further research on minimising loss and characterising metal uptake is recommended when using the optimised chronic test protocol to assess metal toxicity.

While acute toxicity test data play an important role in risk assessment processes, in Australia and New Zealand the preferred method for deriving GVs continues to be based on the SSD approach, using chronic effects data, such as EC10 or NOEC values (Warne *et al*. 2018). Single-toxicant and single-species chronic effects data from laboratory toxicity tests are the main source of data used in SSDs (ANZG 2018). Where chronic effects data are insufficient, acute data may be converted using an ACR previously determined for a species, but reliability of the SSD may be reduced (Warne *et al*. 2018).

The acute and chronic test protocols optimised in this research may also be used for the protection and management of other tropical mussels throughout Northern Australia, where anthropogenic activities such as mining, or agriculture have been identified as having a potential impact on mussel populations. While the chronic ammonia data generated from this study have already been used to inform the derivation of a site-specific GV for ammonia (Supervising Scientist 2018c), further data may be generated for other contaminants of potential concern, such as manganese, aluminium, cadmium, chromium, and sulfate using the optimised test protocols. Acute and chronic toxicity assessments for these contaminants with *Velesunio* spp. will provide valuable data to contribute to the derivation and revision of the site-specific GVs in the AAR.

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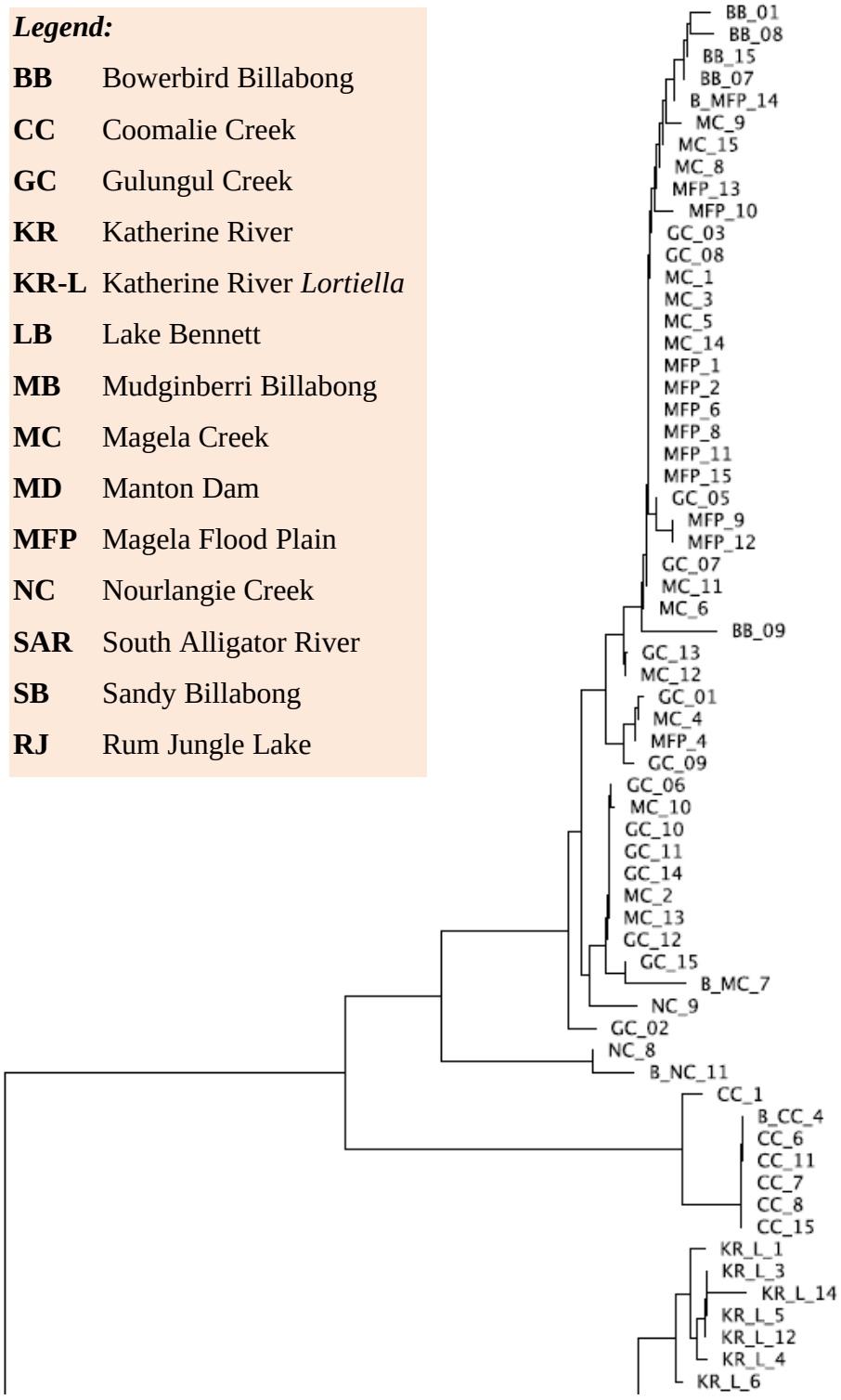
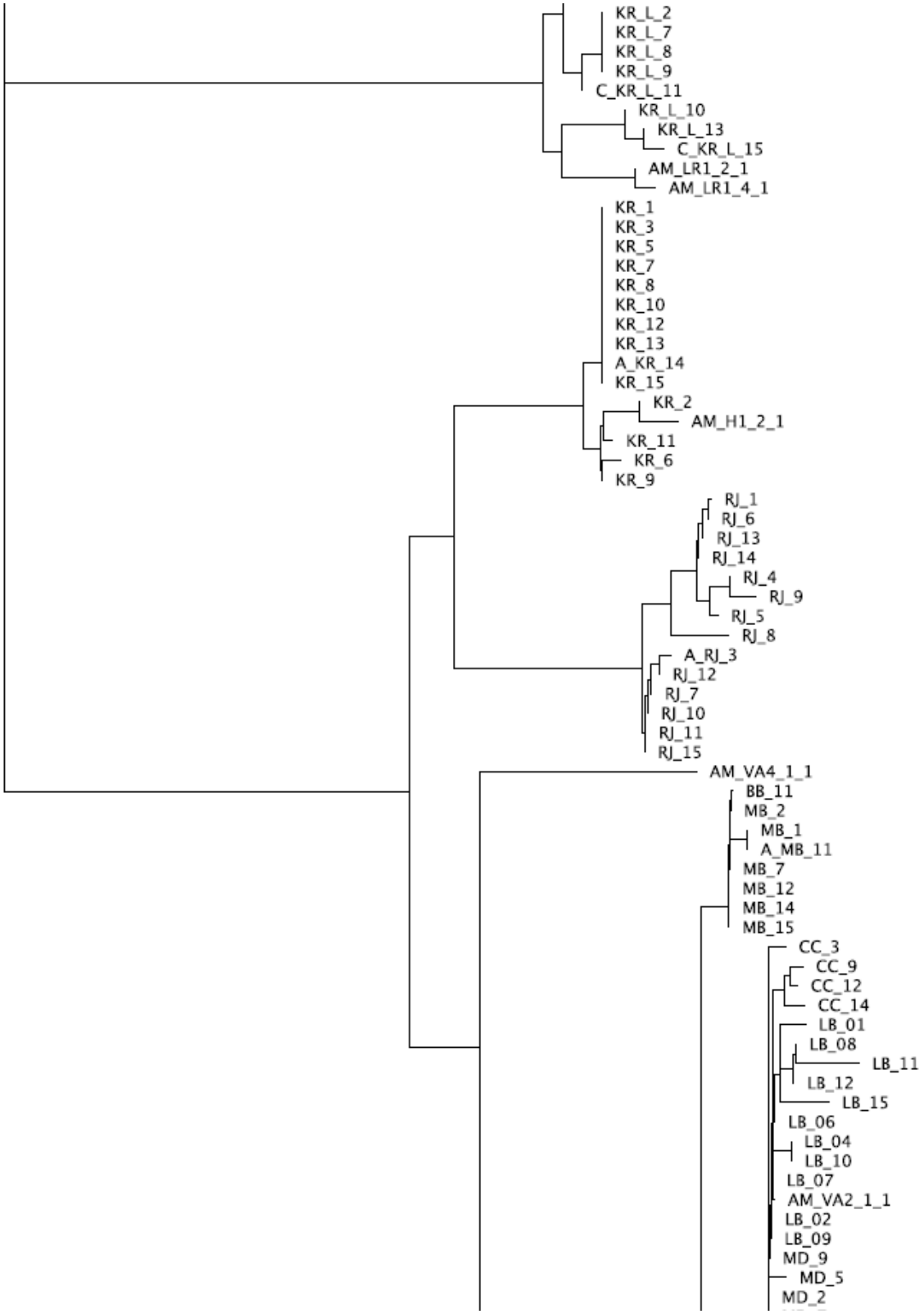
APPENDICES

A. Phylogenetic tree

Legend:

BB	Bowerbird Billabong
CC	Coomalie Creek
GC	Gulungul Creek
KR	Katherine River
KR-L	Katherine River *Lortiella*
LB	Lake Bennett
MB	Mudginberri Billabong
MC	Magela Creek
MD	Manton Dam
MFP	Magela Flood Plain
NC	Nourlangie Creek
SAR	South Alligator River
SB	Sandy Billabong
RJ	Rum Jungle Lake

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RJ_13
RJ_14
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RJ_5
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RJ_12
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RJ_11
RJ_15
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MB_15
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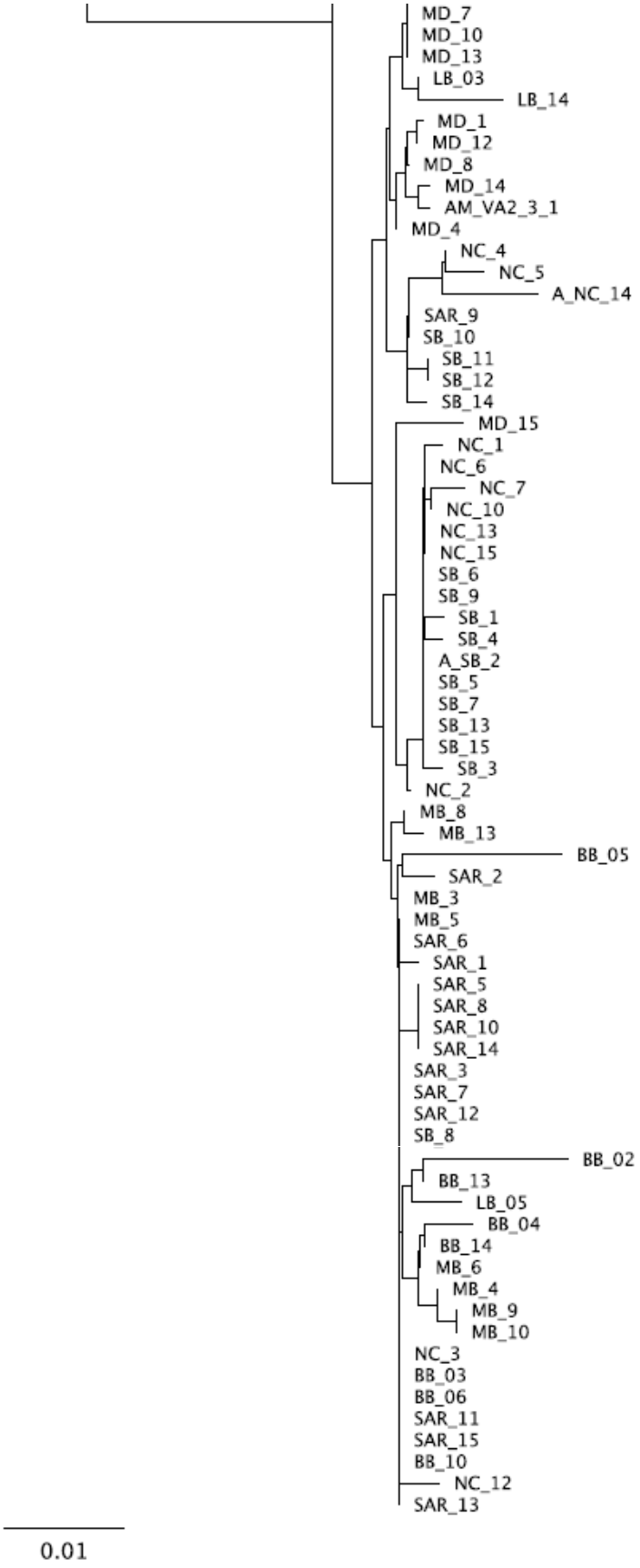
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LB_14
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MD_12
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NC_5
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BB_03
BB_06
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NC_12
SAR_13
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B. Host fish exposure method development

METHODS

The northern trout gudgeon, *Mogurnda mogurnda*, was the main host fish species used to acquire juvenile mussels. *Mogurnda mogurnda* were reared from fry in filtered Darwin tap water (FDTW) and were used as host fish when they reached ~15 cm length (about 6 months old). Another fish species, the chequered rainbowfish, *Melanotaenia splendida inornata*, was sourced from a local supplier (Hurley's Aquariums) and was also trialled for use as a host fish when they reached ~12 cm length (about 6 months old). Both fish species are common in the ARR, and were maintained in laboratory aquaria.

Host fish were exposed to glochidia by placing two *M. mogurnda* or four *M. splendida inornata* into a 5 L plastic beaker containing 4 L of continuously and vigorously aerated FDTW. Densities of glochidia were estimated from the average count of ten 25 µL subsamples of suspended glochidia taken from the known volume of the pooled sample. Measured volumes of glochidia from the pooled sample were added to the beaker to achieve densities of ~12,500 to ~37,500 per fish in the 4 L exposure volume. The fish were exposed for different times (15 to 45 minutes), and the water was agitated by moving the air stone through the water column every 10 minutes to encourage the fish to move, and to ensure glochidia were suspended throughout the water column. The fish were then transferred gently using a wide mesh net (so that any remaining open glochidia were not transferred) into a 75 L plastic tub containing ~60 L of FDTW.

Fish were fed commercial fish pellets daily (Hikari® sinking carnivore pellets, Kyorin co. Ltd, Japan) and one-third of the water was exchanged daily by removing 20 L from the tubs and replacing with fresh FDTW. Material falling from fish into the bottom of tubs was siphoned through a 63 µm stainless steel sieve from day 5 onwards to collect post-excysted or 'released' juvenile mussels. Fish were held in the tubs for up to 12 d initially, with collected juveniles counted daily from first release until last release (approximately 10 d), to determine the period of maximum excystment at ~27 °C. Once this was established, fish were returned to their original holding aquaria after the juveniles required for testing had been collected.

Ten host fish exposures to glochidia were compared to determine optimal exposure and holding conditions for production of juveniles (Table 1). The variables tested or measured were: mussel species, fish species, exposure duration, glochidia density, and peak day of juvenile excystment. *Mogurnda mogurnda* was used exclusively as a host fish for glochidia in eight tests, while two tests compared *M. mogurnda* and *M. splendida inornata* as host fish. For the period of juvenile encystment, mean holding temperatures ranged between 24.5 and 27.8°C.

RESULTS

The number of juveniles produced from ten host fish exposure trials ranged from 25 to 2173 per fish (Table 1). Juvenile numbers increased with increasing exposure duration and initial density of glochidia, and varied more between host fish species than between mussel species and site. In tests 7 and 9, the peak juvenile production for *M. splendida inornata* occurred on day 6 and was over 80% lower per fish than the peak juvenile production for *M. mogurnda* (Table 1) under the same exposure conditions and despite similar size of fish. Additionally, when shell growth rates of juveniles from these two host fish were compared during two 14-d tests (Figure 1), those from *M. mogurnda* had a significantly higher growth rate than those from *M. splendida inornata* (Test 1: 21.0 vs 17.2 µm/day, $P = 0.002$; Test 2: 22.9 vs 16.2 µm/day, $P < 0.001$), and higher survival rate (100% in both tests) compared to that of *M. splendida inornata* (85% and 95% respectively).

Optimal exposure conditions for producing a sufficient number of healthy juveniles for toxicity testing (~300) included a 35-minute exposure duration, agitation every 10 minutes, and a 'medium' density of glochidia (18/mL, Table 1). The peak day of juvenile release occurred on days 7, 8 or 9 following exposure, under temperatures averaging between 24.5 to 27.8 °C (Figure 2, Table 1), and these juveniles were used for the test development and ammonia exposures.

Some fish injuries or death occurred after host fish exposure, either from bullying or fighting between the fish housed together, or in one case from fungal infection. Reductions in stress, injury or death were achieved by housing fish singly in tubs, or by ensuring that only fish of similar sex and size were held together during juvenile encystment.

Table 1: Results of ten host fish exposures under various conditions to produce juvenile mussels using glochidia sourced from both species and four different sites: MC = Magela Creek, GC = Gulungul Creek, SB = Sandy Billabong, LB = Lake Bennett.

Test	Exposure date	Mussel species	Site	# of fish	Fish species	Exposure duration (min)	Glochidia density [a]	Mean temperature during encystment [b] (min – max), °C	Total # of juveniles	Mean # of juveniles per fish	Peak day [c]
1	18/4/16	*Velesunio* sp.	MC	2	*M. mogurnda*	15	Low	27.7 (25.1 – 29.6)	50	25	9
		Velesunio sp.	MC	2	*M. mogurnda*	30	Low	27.7 (25.1 – 29.6)	148	74	8
2	14/5/16	*Velesunio* sp.	MC	4	*M. mogurnda*	40	High	27.8 (23.0 – 31.5)	8694	2173	8
3	25/11/16	*V. angasi*	SB	4	*M. mogurnda*	35	Medium	27.4 (23.5 – 28.9)	2284	571	8
4	11/1/17	*V. angasi*	LB	2	*M. mogurnda*	35	Medium	27.4 (24.9 – 28.8)	2542	1271	8
5	11/2/17	*V. angasi*	LB	2	*M. mogurnda*	35	Low	27.6 (25.3 – 28.9)	885	443	8
6	5/3/17	*Velesunio* sp.	GC	2	*M. mogurnda*	35	Low	27.5 (25.1 – 28.8)	560	280	7
7	16/3/17	*V. angasi*	LB	2	*M. mogurnda*	35	Medium	27.4 (25.1 – 28.8)	1538	769	8
	16/3/17	*V. angasi*	LB	4	*M. splendida*	35	Medium	27.4 (25.1 – 28.8)	570	143	6
8	20/4/17	*Velesunio* sp.	GC	4	*M. mogurnda*	35	Low	25.7 (25.2 – 26.3)	923	230	9
9	28/5/17	*V. angasi*	LB	2	*M. mogurnda*	35	Medium	24.5 (22.4 – 25.9)	1475	738	8 & 9
	28/5/17	*V. angasi*	LB	4	*M. splendida*	35	Medium	24.5 (22.4 – 25.9)	508	127	6
10	22/10/17	*V. angasi*	SB	2	*M. mogurnda*	35	Medium	27.5 (24.5 – 29.3)	2420	1210	9

[a] Glochidia density represents approximate numbers in 4 L water: Low = 25,000 (6/mL), Medium = 75,000 (18/mL), High = 100,000 (25/mL).

[b] Temperature of laboratory where tubs were held, measured using a data logger (Testo Saveris™)

[c] Peak day: the day/s of maximum juvenile excystment after host fish exposure on day 0.

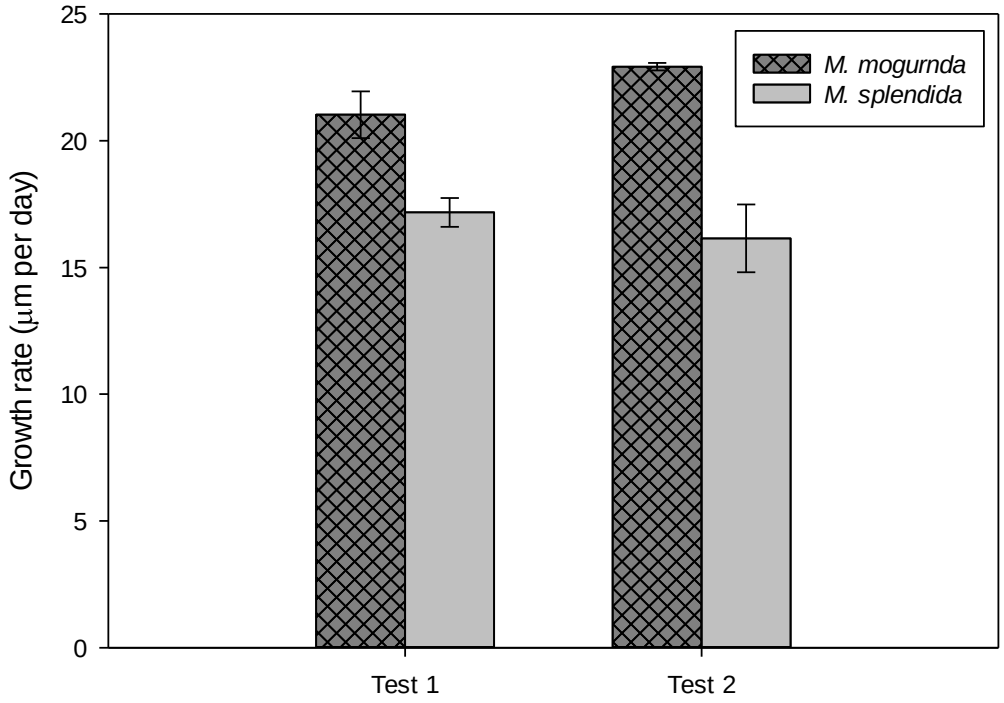

Figure 1: Comparison of growth rates (± SE between two replicates) in two tests between juvenile mussels metamorphosed on gudgeons (*Mogurnda mogurnda*) and rainbowfish (*Melanotaenia splendida inornata*) during host fish exposures (#9 and #10, Table 1). Juveniles were grown for 14 days in Magela Creek Water with added sediment achieving turbidity of 100 NTU and fed green alga *Chlorella* sp. at ~8 x 10^4 cells/mL throughout each test. Shell length was measured on days 8 and 14.

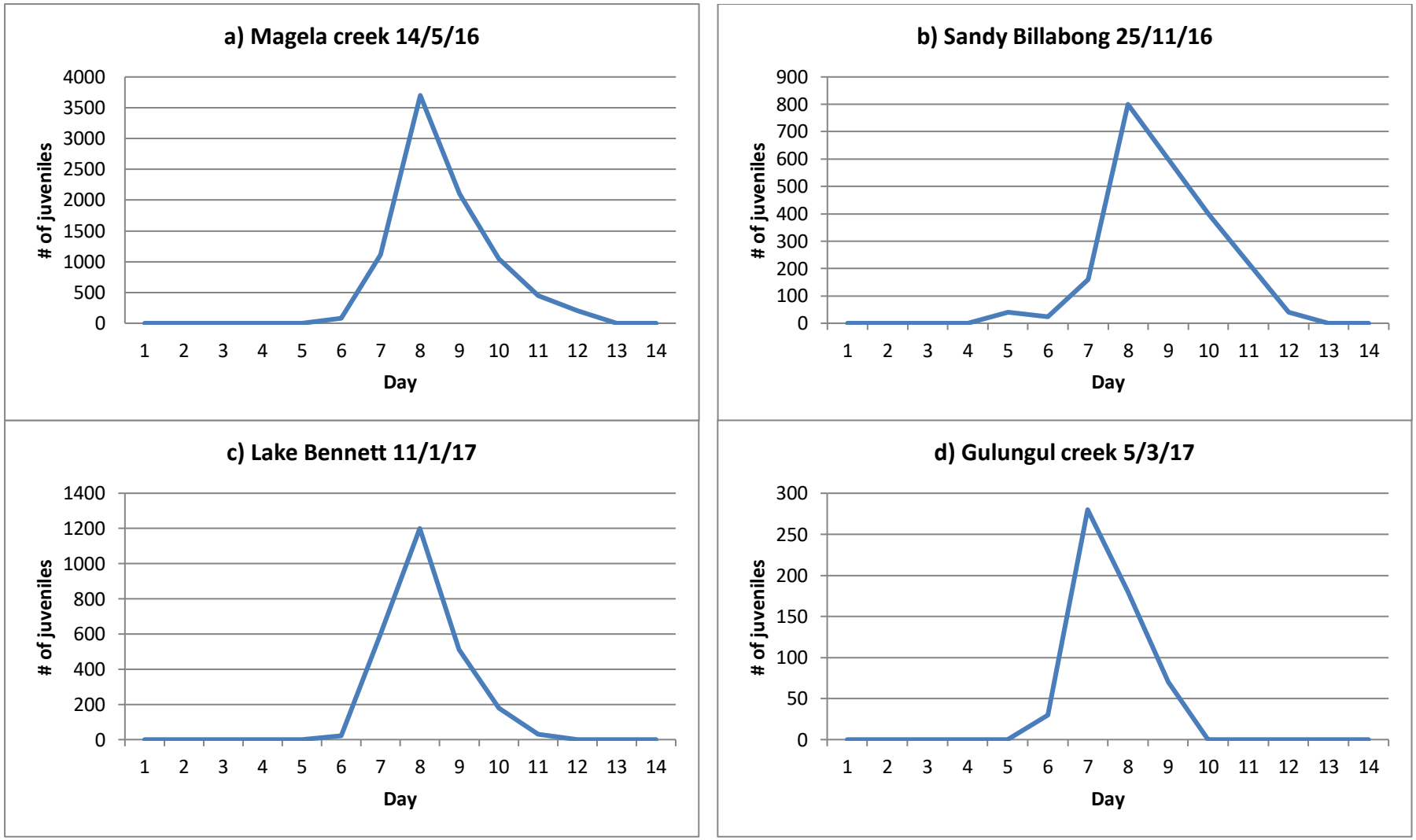

Figure 2: Results of host fish exposures using mussels from four different collection sites, showing the peak day of juvenile excystment after glochidia were exposed to host fish.

DISCUSSION

Mogurnda mogurnda was a more suitable host-fish than *Melanotaenia splendida inornata*. Whilst some unionid mussels have exhibited a high level of host-fish specificity (Zieritz *et al*. 2016), *Velesunio* spp. are non-specific parasites of their host-fish, with 19 host-fish species (including *M*. *mogurnda* and *M. splendida inornata*) identified in Magela Creek (Humphrey and Simpson 1985). More recently, another host-fish, the Hyrtl's tandan (*Neosilurus hyrtlii*), was identified for *Velesunio angasi* in the Pilbara region of Western Australia (Klunzinger *et al*. 2010). In the present study, the number of glochidia encysted per fish on *M. splendida inornata*, was ~20% of those encysting *M. mogurnda*. This is in agreement with Humphrey and Simpson (1985) who observed that benthic-dwelling fish species that were notably inactive in the water column experienced the highest incidence and intensity of infection. *M. mogurnda* was clearly an inactive bottom-dwelling fish during this study, while *M. splendida inornata* was highly active in the water column. These behaviours may explain the higher incidence of glochidia infection on *M. mogurnda,* as glochidia sank to the bottom of the beaker during host-fish exposures and required agitation to keep suspended. However, the lower infestation of glochidia on *M. splendida inornata* could also have resulted from the fish consuming some of the glochidia during exposure, as observed when glochidia were first added to the beaker. Juvenile health and development are influenced by many variables, such as temperature, intensity of glochidia infection, and health of the fish used. Humphrey and Simpson (1985) reported that the duration of the parasitic period of *V. angasi* glochidia was temperature dependent, recording metamorphosed juveniles from the host fish, *Glossogobius giurus*, sooner (5 d average) at 30°C than those at 22°C (10 d average). In the present study, the development rate of juveniles was similarly fast (8 d average) in the high water temperatures used (26.9 average), with the earliest metamorphosed juveniles from *M. mogurnda* collected 5 days after infection, and the earliest from *M. splendida inornata* collected 3 days after infection. Whilst *M. splendida inornata* successfully produced juvenile mussels, the vigour and health of the mussels were reduced compared to those collected from *M. mogurnda*, indicating that while *Velesunio* spp. may be non-specific host-fish parasites, some host-fish are less effective, resulting in lower infestation rates and reduced health of the excysted juveniles. The host-fish exposure method for acquiring sufficient numbers of healthy juveniles for toxicity testing (i.e. ~300) was optimised, whilst minimising stress to the host-fish by over-exposure at initial infection.

C. Methods for the chronic toxicity test optimisations

Test volume and test vessels

To determine optimal test volume and test vessels, four different test volumes and three different test vessels were trialled during four separate tests: 30 mL in plastic petri dishes with lids (diameter ~8.5 cm, water height ~0.7 cm), 50 mL in plastic jars with lids (diameter ~4.1 cm, water height ~3.7 cm), and 100 mL and 150 mL in plastic jars with lids (diameter ~6.0 cm, water height ~3.1 or ~4.7 cm respectively). Treatments were duplicated and seeded with ten juvenile mussels. Green alga (*Chlorella* ~8 x 10^4 cells/mL) was used as the food source for all tests except for one that also included treatments comparing Shellfish Diet as the food source (Test a). Sediment for sustaining growth and survival (see '*sediment and feeding'* below) was provided at turbidity of 100 NTU (Tests a and d) or 200 NTU (Tests b and c). From these test results, the optimal test vessel was selected, and a final growth test was run in triplicate using Lake Bennett mussels (*V. angasi*) with 100 NTU and a range of volumes (30 – 150 mL) to confirm the optimal test volume. Containers were not aerated during the trials.

Sediment and feeding

Sediment collected from the edges of Sandy Billabong (a natural, non-mine disturbed, backflow billabong: latitude 12° 54' 4''S, longitude 132° 46' 38''E) was wet sieved, retaining the < 63 µm fraction. The resulting liquid fine sediment (~80% water content) was placed in 40 mL sealed plastic vials to eliminate evaporation and heated in a 60°C oven for 48 h to reduce risk of pathogens, then stored at -4°C until required for testing. Sediment concentrations in the tests were not directly measured. Instead, turbidity was used as a proxy for suspended sediment. At the commencement of each test, sediment was initially present in suspended form. However, settling occurred after a short period (three to four hours) until the sediment had formed a thin coating on the bottom of the test vessels. A range of starting turbidity values (0, 25, 50, 75, 100, 200, 225 and 500 NTU) were trialled during three experiments to determine the minimal amount of fine sediment that supported optimal juvenile growth, and a final combined feeding and turbidity trial was conducted co-factorally (see Chapter 3, Figure 4.2). The ASTM (2006) do not specifically recommend a feeding regime for longer term toxicity tests with juvenile mussels, citing various food sources used in tests longer than 10 d, which used monocultures or mixtures of commercial or laboratory cultured algae. Three different food sources were tested during this study: 1) Shellfish Diet®, a commercially-available algae mix (Reed 2012) consisting of six marine microalgae

(*Isochrysis*, *Pavlova*, *Tetraselmis*, *Chaetocerous calcitrans*, *Thalassiosira weissflogii* and *Thalassiosira pseudonana*) ranging in size from 4 – 20 µm; 2) the unicellular green alga, *Chlorella* sp. (~2 µm), which was cultured in-house and originally sourced from the Magela Creek system in 1991 (Riethmuller *et al*. 2003); and 3) Fermented Food with Vitamins (FFV), a nutrient/bacterial mixture used to feed cladoceran test species. The FFV was cultured in-house by fermenting ground fish food, alfalfa, vitamin B12 and vitamin B5 (Riethmuller *et al*. 2003).

Trials were run with the algal food sources (Shellfish Diet and *Chlorella* sp. above) to determine an optimal cell density for juvenile growth. FFV was trialled only as a supplement to Shellfish Diet and *Chlorella* sp., using the same concentration as that used for cladoceran feeding (1 µL/mL). Shellfish Diet (cell density 2 x 10^9 cells/mL) was diluted 1:10 with MilliQ water to reduce viscosity and achieve a lower cell density based on the feeding rate recommended by the manufacturer (mL/day = wet weight of juveniles including shell x 0.036). Using this formula, a median feeding volume and feeding rate of 40 µL (cell density 8 x 10^4 cells/mL) every two days of diluted Shellfish Diet per replicate of 10 mussels was determined for testing. Cell densities ranging from 2 x 10^4 to 2 x 10^5 cells/mL were added to each 100 mL replicate at test initiation and during each water change every two days, and growth after 14 d was compared to determine the cell density which produced the highest growth rate. The corresponding median algal cell density for Shellfish Diet (8 x 10^4 cells/mL) was used as the median for trials using *Chlorella* sp. Cell densities were measured using flow cytometry (Accuri C6, BD Biosciences). Once *Chlorella* sp. was chosen as the food source, a final combined feeding and turbidity trial was conducted co-factorally using three different cell densities of *Chlorella* sp. (3.2 x 10^4, 7.6 x 10^4, and 1.3 x 10^5 cells/mL), each at three different turbidities (50, 100 and 200 NTU) to confirm the optimal feeding density and turbidity.

Water changes and pH control

The frequency of water changes was investigated to determine the minimum frequency with no adverse effects on growth or water quality. The water-change frequencies tested were: i) days 4, 8 and 12; ii) day 7 only; or iii) every second day. For i) and ii), only one replicate per treatment was used due to the low numbers of juveniles available at the time of the experiment. Tests were run using 100 NTU of sediment and 3.9 x 10^8 cells/mL of *Chlorella* sp. Daily water changes were not trialled due to the large amount of water that would be required, and because acceptable water quality was still maintained with less frequent (than daily) changes. During water changes, juveniles were removed from the old

water using a plastic pipette and transferred into new water. In this way, accumulating sediment could be removed at each water change. A method of pH control was necessary when testing with ammonia, due to its influence on ammonia toxicity. Magela Creek water has an average pH of ~6.0, which was maintained throughout tests using a buffer. Two buffers were trialled: 1) HEPES (N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid) and 2) MES (4-Morpholineethanesulfonic acid, Sigma-Aldrich). Buffers were tested at 1 mM and pH 6.0 with optimal growth, survival and water quality the criteria used to determine the most suitable buffer.

Endpoints

Three endpoints were assessed in either developmental work or during toxicity testing: 1) shell growth rate; 2) survival; and 3) dry weight (including shell). To measure shell growth rates, mussels were photographed on days 0, 8, and 14 using a camera connected to a microscope (Leica MC170HD camera, Leica M205C microscope at 8.0 x magnification). The maximum shell length of each mussel was measured between the anterior and posterior ends, using image analysis software (Leica application suite, V4.6.1). To provide a starting average length measurement (day 0), a separate sub-sample of 30 juveniles was measured to avoid handling stress on fragile newly metamorphosed juveniles. For day 8 measurements, juveniles from each replicate were removed from their test vessels during the water change using a 2 mL plastic pipette, then placed into a 6-well plate containing the corresponding test water solution taken from near-surface waters of each test vessel. Excess sediment in the 6-well plate was removed using a pipette, and mussels were photographed before placing into new water replicates. For day 14 (end of test) measurements, mussels were placed into a 6-well plate containing 5 mL of MCW per well. In later tests, ethanol (70%) was used as a euthanising agent to prevent mussel movement, enabling photographs to be taken faster. Survival was recorded during water changes, and assessed by classing juveniles with closed valves, opaque appearance, the presence of internal organs, and a moving foot as alive. Dead individuals were identified as empty shells or opened shells with no internal movement. Dry weight was investigated as an additional endpoint in a food and turbidity test (see Chapter 4, Supplementary Figures S4.5 – S4.8) by drying each replicate of 10 mussels (after end of test growth measurements) in separate pre-weighed aluminium trays for 72 h and weighing the tray and dried sample (Sartorius CPA225D). The composite dry weight of surviving mussels was used to calculate the weight per mussel from each treatment, then compared to the shell growth rate endpoint to determine the most reliable endpoint.

D. Methods for pH adjustment to compare data

1. The Emerson *et al*. (1975) method uses the following equations to calculate the percentage of un-ionised ammonia in solution, as a function of pH and temperature:

(1) $$pKa = 0.0901821 + 2729.92/T$$

(2) $$f = 1/ [10^{(pKa-pH)} + 1]$$

where, pKa = dissociation constant at any temperature
T = temperature in Kelvin (K), T(K) = 273.15 + T(°C)
f = fraction of un-ionised ammonia (multiply by 100 for percent)

These equations are based on the assumption that un-ionised ammonia (NH3) is much more toxic than the ammonium ion ($NH4^+$). They can be used to calculate the proportions of NH3 and NH4+ in a solution at any pH and temperature.

2. The USEPA method of normalising chronic ammonia values to a standard pH and temperature follows several steps:

a) If chronic values were reported in unionised ammonia concentrations, they were converted to Total Ammonia Nitrogen (TAN) at the reported pH and temperatures, using the pKa relationship from Emerson *et al.* (1975) above, equation (1).

b) Chronic TAN values were then adjusted to pH 7 using the pH relationship described in USEPA (2013):

(3)

$$CV_{t,7} = \frac{CV_t}{\left(\frac{0.0278}{1 + 10^{7.688-pH}} + \frac{1.1994}{1 + 10^{pH-7.688}}\right)}$$

c) After adjustment to pH 7, the chronic TAN values were further adjusted to a temperature of 20°C (invertebrates only), using the following equation:

(4)

$$\log (CVt, 7, 20) = \log (CVt, 7) - [-0.028(\text{test temperature in °C} - 20°C)]$$

These pH- and temperature-dependent equations were developed with the assumption that the toxicity of ammonia is influenced by the joint toxicity of un-ionised ammonia and ammonium ion. The equation is applicable from pH 6 to 9, but there is uncertainty at the lower end of this range due to increasing scatter of the data, and extrapolation below pH 6 is not advised (USEPA 2013).

E. Animal Ethics permission

Animal Ethics Committee
Certificate of Project Approval

This is to certify that the animal research project:

A15018

Development of a toxicity test for the assessment of ammonia toxicity using the native freshwater mussel, Velesunio angasi

Australian Government, Department of Environment, Supervising Scientist Division - 003 Exp 06/2017

has been granted approval by the CDU AEC for the period:

21 January 2016 to 21 January 2019

Using no more than:

Northern Trout Gudgeon *(Mogurnda mogurnda)* **12 per annum**
Checkered Rainbowfish *(Melanotaenia splendida)* **6 per annum (Year 1); 12 per annum (Year 2 & 3)**
Mouth Almighty *(Glossamia aprion)* **6 per annum (Year 1); 12 per annum (Year 2 & 3)**
Sailfin perchlets *(Ambassus ssp)* **6 per annum (Year 1); 12 per annum (Year 2 & 3)**

Animal Research Permits

The AEC has issued the following Animal Research Permits under Section 48 of the *Animal Welfare Act* to the following investigators:

Principal Investigator

Dr Andrew Harford

Other Investigators

Dr Thomas Mooney
Mrs Claire Costello
Ms Linda Kleinhenz
Dr Melanie Trenfield
Mr Ceiwen Pease

Animal Research Permits remain in force for two years after the date of approval. Renewal permit applications (i.e. Form A and B Declarations) must be submitted with Progress Report (Year 2).

Special Conditions of Approval

Location: Ecotoxicology Laboratory, Environmental Research Institute of the Supervising Scientist (*eriss*), Eaton, NT

AEC Inspections

Annual inspections of research projects and associated animal facilities are obligatory and documentation must be available to the AEC upon request.

Signature
Issued by Professor Keith Christian,
Chair of the Charles Darwin University Animal Ethics Committee
on behalf of the Charles Darwin University